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Engineering *E. coli* to target bladder and colon tumor cells and characteriazation of the adhesion process.

Doctoral thesis

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Abbreviations

aa	amino-acid
Ab	Antibody
Ap	Ampicillin
APEx	Anchored periplasmic expression
AT	Autotransporter
BAM	β-barrel assembly machinery
BCA	Bicinchoninic acid
BCG	Bacillus Calmette-Guerin
BDEPT	Bacterial-directed enzyme prodrug therapy
bp	base pair
CD	Cytosine deaminase
CDR	Complementarity determining region
CH	Constant domain of the heavy chain of an immunoglobulin
CL	Constant domain of the light chain of an immunoglobulin
Cm	Chloramphenicol
c-di-GMP	Bis-(3'-5')-cyclic dimer guanosine monophosphate
DAPI	4',6-diamidino-2-fenindol
DEPC	Diethylpyrocarbonate
DGC	Diguanylate cyclase
DMEM	Dulbecco´s modified Eagle´s medium
DNA	Deoxyribonucleic acid
DNAse	DNA ribonuclease
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EHEC	Enterohaemorrhagic Escherichia coli
ELISA	Enzyme-linked immunosorbent assay
EPEC	Enteropathogenic Escherichia coli
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
Fc	Crystallizable constant fragment of Ab
Fib	Human Fibrinogen

Fv	Variable fragment of an Ab
GFP	Green fluorescent protein
GIT	Gastrointestinal tract
h	hour
HCAb	Heavy chain only Ab
IBD	Inflammatory bowel diseases
Ig	Immunoglobulin
IM	Inner membrane
IPTG	Isopropylthio-β-D-galactoside
kDa	kiloDalton
Km	Kanamycin
LB	Luria-Bertani medium
LPS	Lipopolysaccharide
mAb	Monoclonal Ab
mRNA	messenger RNA
μg	microgram
μl	microliter
μm	micrometer
μM	micromolar
mg	milligram
ml	milliliter
mm	millimeter
mM	millimolar
min	minute
MOI	Multiplicity of infection
nm	nanometer
OD_{600}	Optical density at 600nm
OMV	Outer membrane vesicle
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PG	Peptidoglycan
POD	Peroxidase
RBS	Ribosome binding site

RNA	Ribonucleic acid
RNAse	RNA ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
SA	Synthetic adhesin
scFv	single chain fragment variable
sdAb	single domain antibody
SDS	Sodium dodecyl sulphate
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
T5SS	Type V secretion system
TE	Tris-EDTA
TEM	Transmission electron microscopy
Tir	Translocated intimin receptor
TirM	Extracellular domain of Tir
TK	Thymidine kinase
tm	transmembrane
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
tRNA	transfer RNA
VH	Variable domain of the heavy chain of an Ig
VHH	Variable domain of the heavy chain of a HCAb, also termed Nanobody
VL	Variable domain of the light chain of an Ig
WT	Wild type

Summary

Current therapies against cancer have limited efficacy due to the lack of specificity, high toxicity and poor penetration in tumor tissue. The natural ability of bacteria to colonize and penetrate deep in tumors makes them potential vehicles for delivery of therapeutic molecules. Synthetic biology may allow the profound engineering of bacteria to improve tumor colonization, specificity, reduce bacterial toxicity, and make precision delivery of a payload of therapeutic molecules.

In this PhD thesis, we have conducted research aimed to engineer Escherichia coli (E. coli) bacteria using synthetic biology for precise adhesion and invasion of tumor cells, as well as for the delivery of protein payload in the cytoplasm of the tumor cell. In addition, we have investigated the influence of bacterial motility for specific tumor cell adhesion.

First, we developed synthetic adhesins (SAs) that enable us to program the adhesion of E.coli bacteria to tumor cells expressing human epidermal growth factor receptor (EGFR), a surface receptor tyrosine kinase upregulated on many epithelial tumors such as bladder and colon carcinomas. SAs display a single domain antibody on the bacterial surface that specifically binds a target antigen expressed on the tumor cell surface (e.g. EGFR). We generated SAs binding human EGFR and showed their correct surface display expression in E. coli upon chromosomal integration under the control of a constitutive promoter. We demonstrate that these modified bacteria attach specifically to human bladder and colon epithelial tumor cell lines expressing EGFR.

Next, we engineered the specific bacterial invasion of the targeted tumor cells by inserting, in the chromosome of bacteria expressing SAs, the gene encoding the invasin (Inv) protein from Yersinia pseudotuberculosis under the control of an inducible promoter. Further, we tested Inv expression in combination with the pore-forming protein Lysteriolysin O (LLO) from Listeria monocytogenes to elicit the release of a protein cargo in the cytoplasm of the targeted tumor cells.

Synthetic adhesins against EGFR were also expressed in an E. coli strain producing minicells, non-live bacterial nanoparticles devoid of chromosomal DNA, that may represent an interesting alternative to the use of live bacteria for tumor targeting. We produced minicells simultaneously expressing SAs against EGFR on their surface and a cargo protein in their cytosol. However, we found that adhesion of these minicells to EGFR-positive tumor cells was very poor compared with that of live bacteria expressing an identical SA. This result prompted us to investigate this

difference and lead us to discover the critical role of an active flagellar motility for efficient adhesion of E. coli bacteria to the target tumor cells.

E. coli mutants lacking flagella or having a non-motile flagella are not able to bind tumor cells despite expression of SAs. This adhesion defect of non-motile bacteria is not compensated by bacterial movement in unidirectional flow or by centrifugation to force cell contact. We found that after an initial cell contact, an E. coli bacterium must establish simultaneously several adhesion points with the surface of the target cell in order to form a permanent adhesion event. Flagellar rotation in a single direction, either clock-wise or counter clock-wise, is sufficient to generate these local movements and to establish a permanent adhesion, which is characterized by the absence of bacterial motility. We found that the surface sensing pathway mediated by the lipoprotein NlpE and the flagellar "brake" protein YcgR, which binds to cyclic-di-GMP second messanger, are not needed for the permanent adhesion of bacteria to tumor cells.

Lastly, we have extended our conclusions with SAs in E. coli to the adhesion of pathogenic bacteria to mammalian host cells during infection. We have demonstrated that flagella are also required for bacterial adhesion mediated by natural adhesins present in uropathogenic E. coli and Salmonella enterica sv. typhimurium.

Resumen

Las terapias actuales contra el cáncer poseen una eficacia limitada debido a la falta de especificidad, a su alta toxicidad y a su limitada capacidad de penetración en los tumores. La capacidad natural de las bacterias para colonizar y alcanzar áreas profundas del tumor las hace potenciales vehículos para la liberación de moléculas terapéuticas de manera eficiente. La biología sintética nos permite la modificación de las bacterias para mejorar la colonización de los tumores, aumentar la especificidad, reducir la toxicidad, y realizar una liberación precisa de moléculas terapéuticas en el interior del tumor.

En esta tesis doctoral, la investigación ha sido dirigida a la modificación genética de la bacteria Escherichia coli (*E. coli*) utilizando biología sintética para conseguir una adhesión e invasión específica de las células tumorales, así como la liberación de una proteína "cargo" de interés en el citoplasma de la célula tumoral. Además hemos investigado la influencia de la motilidad bacteriana durante la adhesión específica a la célula tumoral.

Primero, desarrollamos adhesinas sintéticas (SAs) que permiten la adhesión programada de E.coli a células tumorales que expresan el factor de crecimiento epidérmico (EGFR), un receptor tirosina-quinasa sobre-expresado en muchos tumores epiteliales humanos como por ejemplo los carcinomas de vejiga y colon. Las adhesinas sintéticas exponen en la superficie bacteriana un anticuerpo monodominio que se une específicamente a un antígeno expresado en la superficie de la célula tumoral (p.ej. EGFR). Hemos generamos SAs capaces de unir EGFR y se ha demostrado su correcta expresión en la superficie de *E. coli* tras su integración en el cromosoma bajo el control de un promotor constitutivo. Hemos demonstrado que las bacterias modificadas mostraban una adhesión específica a células tumorales humanas de vejiga y colon que expresan EGFR.

A continuación se generó la invasión específica de estas células tumorales insertando en el cromosoma de la cepa modificada con SAs, el gen que condifica a la proteína invasina (Inv) de Yersinia pseudotuberculosis bajo el control de un promotor inducible. Además, se testó la expresión de la invasina en combinación con la proteína formadora de poros Listeriolisina O (LLO) de *Listeria monocytogenes* para producir la liberación de una proteína "cargo" en el citoplasma de las células tumorales.

Las adhesinas sintéticas contra EGFR también fueron expresadas en una cepa de *E. coli* productora de minicélulas, partículas bacterianas no vivas carentes de cromosoma, que pueden representar una alternativa interesante al uso de bacterias vivas frente a tumores. Hemos generado minicélulas expresando SAs que reconocen EGFR y una proteína "cargo" en el citoplasma. Sin embargo, en nivel de adhesión de estas minicélulas a las células tumorales que expresan EGFR fue muy bajo en comparación con las bacterias vivas expresando las mismas SAs. Este resultado nos condujo a estudiar las diferencias entre bacterias y minicélulas que podían explicar su distinto nivel de adhesión y descrubrimos la importancia de la motilidad bacteriana para una adhesión efectiva de *E. coli* a la célula tumoral diana.

Los mutantes de *E. coli* sin flagelo, o que expresan un flagelo no motil, no se unían a las células tumorales a pesar de expresar las SAs. Esta baja adhesión de las bacterias no motiles no se podía compensar mediante el movimiento de las bacterias en un flujo unidireccional o por centrifugación para forzar el contacto con la célula. Descubrimos que tras un contacto inicial, *E. coli* debe establecer simultáneamente varios puntos de adhesión con la superficie de la célula diana para crear una adhesión permanente. La rotación flagelar en una sola dirección, a favor de las agujas del reloj o en contra de las agujas del reloj, es suficiente para generar estos movimientos locales y para establecer la adhesión permanente de la bacteria, que se caracteriza por la ausencia de movimiento de la bacteria. También observamos que ni la ruta para sensar superficies mediada por la lipoproteína NlpE, ni la proteína “freno” YcgR, que une el dinucleotido cíclico de guanosina difosfato (cyclic-di-GMP), son requeridas para la adhesión permanente de la bacteria a las células tumorales.

Finalmente, hemos extendido nuestras conclusiones obtenidas con *E. coli* expresando SAs pueden aplicarse a la adhesión de bacterias patógenas a las células del huesped mamífero. Hemos demostrado que el flagelo es necesario para la adhesión mediada por adhesinas naturales presentes en *E. coli* uropatógenas y en *Salmonella enterica sv. typhimurium.*

Objectives

-Characterization of the binding affinity and epitope recognition of anti-EGFR nanobodies selected by *E. coli* display for their application in synthetic adhesins.

-Generation of engineered *E. coli* strains constitutively expressing synthetic adhesins anti-EGFR for targeting bladder and colon epithelial tumor cells.

-Engineer *E. coli* strains targeting EGFR-positive epithelial tumor cells with invasive capabilities for the specific release of a protein cargo in the tumor cell.

- Evaluate the potential of synthetic adhesins anti-EGFR expressed in non-live *E. coli*-derived minicells for targeting epithelial tumor cells and release of a protein cargo.

- Characterize the role of flagella in the adhesion of *E. coli* to tumor cells.

Introduction

1. The use of bacteria fighting cancer.

Bacteria were first explored as an alternative cancer treatment nearly 150 years ago. The first doctor using on purpose bacteria as an anti-tumor agent probably was the German physician W. Bush in 1868, inducing a bacterial infection in a patient presenting sarcoma (Pawelek et al., 2003). Almost 30 years later the American physician William Coley was the first to experiment with bacteria treatments for cancer by injecting his patients presenting sarcoma with various combinations of live and heat-killed bacteria *Streptococcus pyogenes*. Eventually he developed a mixture called Coley´s toxin that caused regression of the majority of tumors, sometimes even the entirely elimination of them (Coley, 1891; Pawelek et al., 2003). However, the lack of knowledge about the mechanism of action of bacteria, together with the inability to control infection in this pre-antibiotic era, as well as the advent of radiotherapy and chemotherapy, relegate this type of treatment (Malmgren and Flanigan, 1955).

Since then, several genera of bacteria have been shown to colonize solid tumors, all of them anaerobic or facultative anaerobic bacteria including *Clostridium* (Nuyts et al., 2002; Theys et al., 2001), *Listeria* (Quispe-Tintaya et al., 2013; Zhou et al., 2018), *Salmonella* (Forbes et al., 2003; Pawelek et al., 1997; Ryan et al., 2009), *Bifidobacterium* (Fujimori et al., 2002; Yazawa et al., 2001) and *Escherichia* (Yu et al., 2004; Zhang et al., 2012). An advantage of facultative anaerobes, such as *Salmonella* and *E. coli*, is that they are highly motile and colonize oxygenated as well as hypoxic and necrotic tumor regions. Nowadays, these observations and the development of synthetic biology have triggered a re-emergence of the use of bacteria as anti-tumor vectors.

1.1 The natural ability of bacteria for colonizing tumors.

The fast growth of tumors require the formation of new blood vessels that provide nutrients and oxygen to the dividing tumor cells. However, tumor neovasculature is disorganized and fenestrated, with areas having poor vascularization, resulting in acidity and nutrient deprivation as well as areas with reduced oxygen concentration inside the tumor. This poor vascularization of solid tumors also makes certain areas less accessible to chemotherapeutic agents and the lack of oxygen makes the tumor cells more resistant to ionizing radiations (Ha et al., 2012). Bacterial therapies have

gained interest to access these therapeutically-resistant tumor areas (St Jean et al., 2008).

Bacteria released in the blood stream can reach the tumor thanks to its leaky neovasculature and proliferate in the hypoxic tumor core due to the absence of immune system cells. It has also been described a specific chemotaxis of bacteria toward necrotic tumor regions, which are rich in amino acids and other complex metabolites (Lyssiotis and Kimmelman, 2017). This exceptional tumor microenvironment explain the preferential growth of bacteria in tumors and not in healthy tissues (St Jean et al., 2008).

Another important characteristic of bacteria for colonizing tumors is their ability of active movement. Motile bacteria can reach the non-vascularize tumor areas thanks to its active motility, being able to access and proliferate in these regions in contrast to the chemotherapeutic drugs, viruses, liposomes and antibodies, which all rely on passive diffusion (Raman et al., 2019; St Jean et al., 2008).

Once in the tumor area, bacteria may cause an anti-tumor effect by various mechanisms, including direct cytotoxicity against cancer cells, competition for limited nutrients, and activation of the immune system (Kaimala et al., 2018).

Bacteria have a large number of endogenous structures, called pathogen-associated molecular patterns (PAMPs), such as flagellin, fimbriae, lipopolysaccharide (LPS), peptidoglycan (PG), and lipotechoic acids, which are recognized by specific receptors present in immune cells (e.g. Toll-like receptors - TLRs- and NOD-like receptors -NLRs) that trigger signalling cascades activating the immune response (Brubaker et al., 2015; Kawasaki and Kawai, 2014). The stimulation of the immune system triggered by bacteria may reverse the immunosuppression presented in late-stage tumors that causes cancer dissemination (Middlebrook and Dorland, 1984).

Metastatic cancers and multidrug-resistant tumors are also susceptible of being treated with a bacterial therapy making microbial therapies able to provide solution to many clinical need not reachable for the traditional treatments (Forbes et al., 2018).

The most clear example of immunotherapy using bacteria is the current treatment for superficial bladder cancer with Bacillus of Calmette Guerin (BCG), an attenuated strain of Mycobacterium bovis (Gandhi et al., 2013) which causes a non-specific stimulation of the immune response against the tumor after instillation of BCG bacteria in the urinary bladder of patient (Kamat et al., 2014).

1.2 Bacterial motility and it role in tumor colonization.

Motility is an important characteristic of bacteria for colonizing tumor (Toley and Forbes, 2012). Bacteria can reach the non-vascularize tumor areas thanks to its motility and proliferate in these regions where traditional antitumor-drugs, viruses, liposomes and antibodies cannot penetrate because they rely on passive diffusion and not in active transport (Raman et al., 2019; St Jean et al., 2008).

The principal organelle for bacterial motility in facultative anaerobes such *E. coli* or *Salmonella* is flagella (Haiko and Westerlund-Wikstrom, 2013). Flagellar motor responds to the chemoreceptor system to redirect swimming toward beneficial environments (Chaban et al., 2015). The chemotaxis system regulates the swimming behavior of the bacteria by controlling the rotational direction of their flagellar motors (Fukuoka et al., 2012). Extracellular stimuli sensed by chemoreceptors are transduced to an intracellular signal molecule, phosphorylated CheY (CheY-P), which switches the rotational direction of the flagellar motors from counterclockwise (CCW) where bacteria swims in linear movement, to clockwise (CW) where bacteria swims in tumble mode (Fukuoka et al., 2012).

It has been described that quiescent tumor cells in the core of solid tumors acts as attractants for *Salmonella* and the chemotaxis is essential for its penetration and accumulation in tumors (Kasinskas and Forbes, 2007). Non-motile *Salmonella* mutants accumulate in solid tumors 26 times less than the wild type strain, showing the essentiality of flagella in tumor colonization (Raman et al., 2019).

Flagellar synthesis is highly regulated process in *E. coli* and related species (Fitzgerald et al., 2014) its expression is regulated by the flhDC protein complex, which control the expression of at least 14 operons encoding more than 40 genes involved in the flagella structure (Liu and Matsumura, 1994). Overexpression of FlhDC increases intracellular accumulation and tumor colonization 2.5 to 5 times more than wild type *Salmonella* (Raman et al., 2019).

The flagellum is made of three major parts: the basal body, the hook and the filament. The basal body works as a reversible rotary motor, and the filament, typically 10–15μm long, serves as a helical propeller. The hook is a universal joint that connects the motor with the filament to transmit torque regardless of the orientation of the filament propeller (Berg, 2003; Chen et al., 2011; Hosu et al., 2016; Macnab, 1999). The flagellar motor is powered by the electrochemical potential difference of hydrogen or sodium ions across the cytoplasmic membrane (Berg, 2003; Minamino et al., 2008). In *Salmonella* and *E. coli*, motor torque is generated by rotor–stator interactions

coupled with proton translocation through the channel formed within the stator. The Mot complex is composed of two cytoplasmic membrane proteins, MotA and MotB, which form a MotA4/MotB2 hetero-hexameric complex (Terashima et al., 2008).

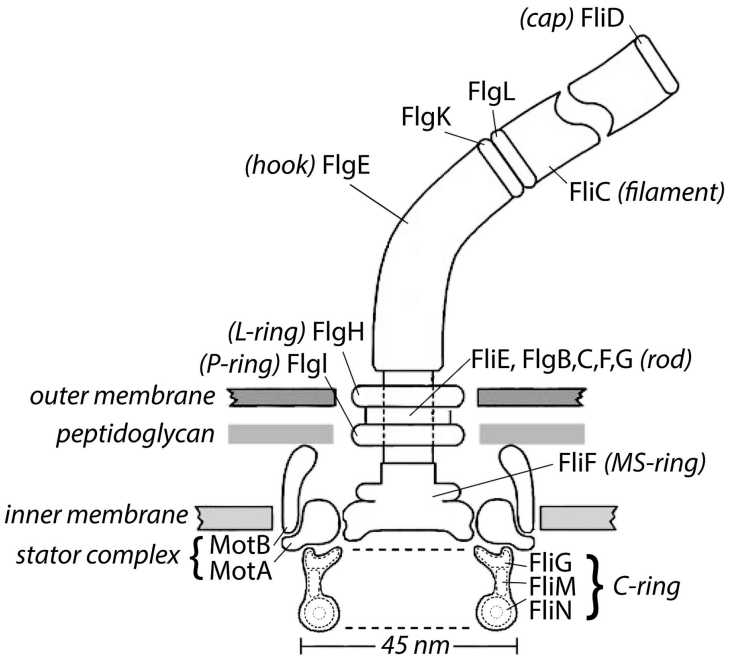


Figure 1. A scale drawing of the base of the E. coli flagellum embedded in three layers of the cell Wall (Hosu et al., 2016).

2. Engineered bacteria as agents to fight cancer

2.1.1 2.1. Reducing bacterial pathogenicity in tumor therapies.

Bacteria can be easily genetically modified to reduce the pathogenicity and increase the anti-cancer effects which comprises another positive point to develop a anti-cancer therapy using those microbes.

An important issue to consider before develop an antitumor-bacterial therapy is the strain of bacteria used. As mentioned above, different bacterial genera have the potential to be used for cancer therapy but all of them have their own unique properties in tumor colonization, their ability to invade tumor tissue and the interaction with the immune system (Chien et al., 2017). Reducing the pathogenicity of bacteria is maybe one of the most important issues to overcome. Bacteria like *Salmonella* or *Clostridium* are very immunogenic, which is appropriate for developing an immunotherapy approach but also may cause a septic shock in case of systemic infection. *Salmonella* strains have received more attention so far as anticancer agents because of the large

understanding of their pathogenicity, physiology and genetics, which drives to defined attenuation strategies.

The most used bacteria are the genetically attenuated VPN20009 and the A1-R strains of *S. typhimurium* . The strain termed VNP20009 from *Salmonella* posses mutations in the genes purI and msbB (lpxM) involved in the synthesis of purines and LPS respectively. Due to the purines auxotrophy the strain grow preferentially inside the tumors (Pawelek et al., 1997) and due to deletion of *msbB* the mutant produce a penta-acylated lipid A of LPS that is weakly recognized by the TLR4 (Wang et al., 2016). VNP 20009 strain had being widely used for tumor targeting to deliver anticancer agents and carrying shRNA- expressing plasmids (Forbes et al., 2018).

The A1-R strain selectively grows in tumor xenografts and was developed by treating *S. typhimurium* with the mutagenic agent nitrosoguanidine (NTG) to induce auxotrophic mutants that were later selected by ability to grow in successive tumor xenografts (Zhao et al., 2005). *S. typhimurium* strain A1 was recovered from this selection and identified as a leucine and arginine auxotroph. Presumably, the auxotrophic strains receive sufficient amounts of these amino acids from the tumor environment but do not persist in the normal tissue environment (Wang et al., 2016). VNP20009 has also been used in clinical trials with modest results (Cunningham and Nemunaitis, 2001; Dramsi and Cossart, 2002). Clinical trials using systemic administration of Salmonella for human cancer therapy revealed that the highest bacterial dose that was tolerated was still insufficient for an effective colonization (Kocijancic et al., 2016; Kramer et al., 2018).

E. coli is extensively used as model host for synthetic biology studies and has the advantage of naturally having non- pathogenic strains that can be used without further attenuation because of that is the second most widely used microorganism in cancer therapy (Chien et al., 2017). There are also *E. coli* strains accepted as probiotics like E. coli Nissle 1917 that have been shown to colonize solid tumors (Danino et al., 2015; Loessner et al., 2009; Stritzker et al., 2007). *E. coli* Nissle had been modified for tumor local delivery of chemotherapeutics (eg. Doxorubycin) (Xie et al., 2017), anti-angiogenic and pro-apoptotic agents like p53 or Tum-5 (He et al., 2019), bacterial toxins (Li et al., 2019), or other molecules such the pro-apoptotic fusion protein TAT-apoptin (Zhou et al., 2011).

2.2 Delivery of anti-tumor molecules by bacteria.

To induce tumor regression and elimination, bacteria can be engineered to express and release cytotoxic proteins such as bacterial toxins (Bishr et al., 2014; Ha et al., 2012; Jiang et al., 2010), pro-drug converting enzymes (Lehouritis et al., 2013), immunoregulatory proteins (Loeffler et al., 2007) and apoptosis-inducing factors (Ganai et al., 2009; Loeffler et al., 2009) that may induce tumor regression and elimination Figure 2.

In the field of the bacterial toxins the most commonly used are the pore-forming cytolysins, such as hemolysin E (HlyE / ClyA) from *E. coli* (Jiang et al., 2010; Ryan et al., 2009) and alpha-hemolysin (SAH) from *Sthaphylococcus aureus* (Bishr et al., 2014; St Jean et al., 2014). Theses proteins are secreted in monomers and polymerize forming pores able to be incorporate into the plasma membrane of mammalian cells (Gouaux, 1998; Henrichsen, 1983). Another interesting strategy used has been the delivery of Flagellin B from *Vibrio vulnificus* that leads to recruitment of macrophages and tumor regression in the tumor area (Zheng et al., 2017).

Bacteria also can be equipped with genes encoding for prodrugs converting enzimes such herpes simplex virus thymidine kinase (TK) or *E. coli* cytosine deaminase (Lehouritis et al., 2013) able to convert a nontoxic compound (i.e. ganciclovir, 5-Fluorocytosine) in a toxic compound or drug (i.e. ganciclovir monophosphate, 5 Fluorouracil) that is going to affect the cells expressing these enzymes or in their vicinity within the tumor area.

Also apoptosis inducing factors and cytotoxic cytokines have been explored such as FAS ligand (FASL), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Tumor necrosis factor alpha (TNFα). These molecules are able to induce apoptosis and are more toxic for tumor cells than for healthy cells (Ganai et al., 2009; Loeffler et al., 2009; Murphy et al., 2017). Local release of these cytotoxic cytokines alleviates their toxicity after systemic administration.

Other cytokines instead of triggering directly tumor cell apoptosis, they are able to activate the host immune system to clear the tumors. IL-2 has been the most extensively used for activating the cytolytic function of the natural killer (NK) cells and promoting lymphocytes activation (Barbe et al., 2005; Ha et al., 2012). IL-18 has also been investigated with this aim (Loeffler et al., 2008).

Genetic transfer to tumor cells of genes encoding for cytotoxic agents (Fu et al., 2008) antiangiogenic factors like endostatin and thrombosponding 1 which kill tumors by preventing the formation of new blood vessels (Liang et al., 2018) or cytokines

genes like IL-12, granulocyte/macrophage colony stimulating factor (GM-CSF) (Yuhua et al., 2001) has been also addresses with tumor targeting bacteria.

Gene silencing has also been achieved by transferring plasmids encoding small hairping RNA (shRNA) from *Salmonella* into cancer cells (Tian et al., 2012).

Gene-specific shRNA are processed into small interfering RNA (siRNA) that induce degradation of the target mRNA, targeting the factor signal transducer and activator of transcription 3 (Stat3) induced remarkably delayed and reduce hepatocellular carcinoma in mice (Tian et al., 2012).

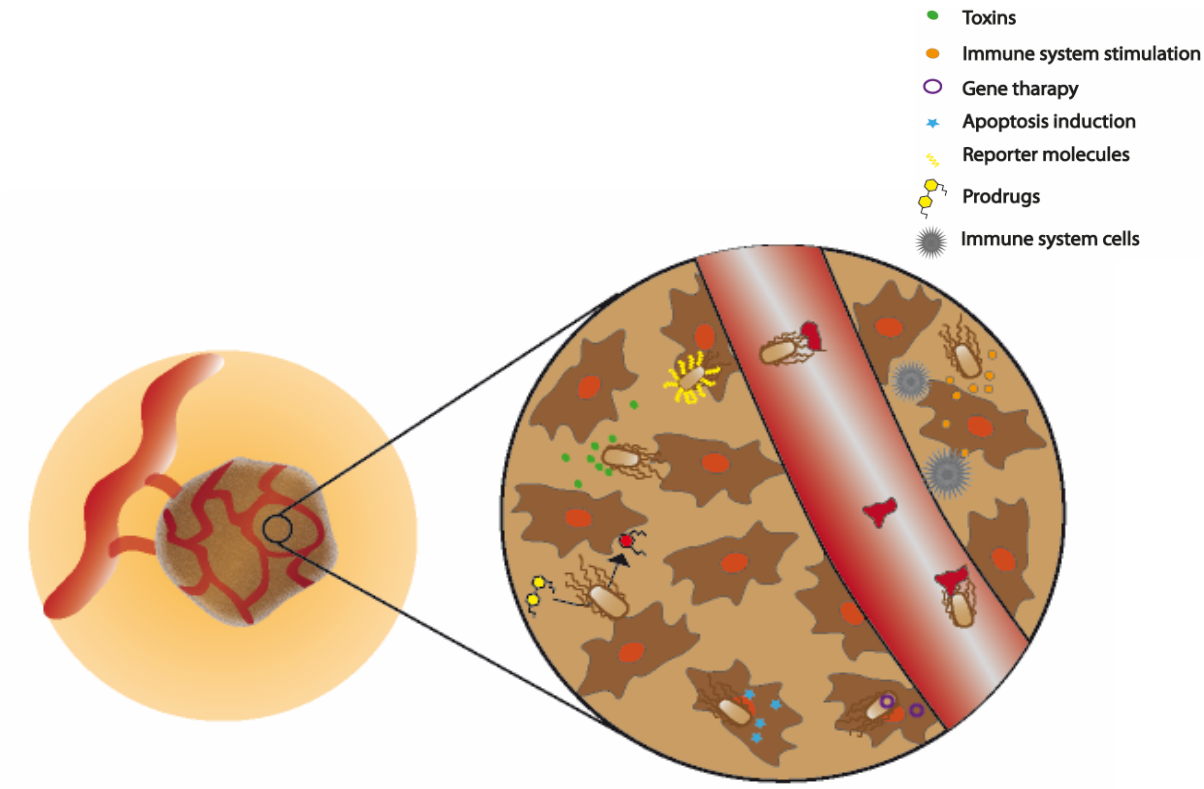


Figure 2. Scheme showing some approaches to improve bacteria as agents in cancer therapies. Bacterial accumulation inside tumors is favored by the leaky vasculature of blood vessels found in tumors (red marks in the blood vessel) The scheme includes expression of reporter genes to follow infection in real time, generally by light emission (yellow wavy lines), localized secretion of bacterial toxins (green dots), bacterial-directed enzyme prodrug therapy (BDEPT) that allows site-specific conversion of prodrugs (yellow molecule) into drugs (red molecule), localized secretion of cytokines (orange dots) to stimulate immune cells (grey cells), intracellular secretion of pro-apoptotic molecules (blue stars) and release of expression vectors (purple circles) coding for different genes or shRNAs.

2.3 Bacterial delivery of therapeutic macromolecules into tumor cells.

The delivery of therapeutic molecules requires release of the macromolecule from the bacteria after colonization of the tumor. Various bacteria delivery systems had been developed for this purpose.

As mentioned above, a simple approach is the release of pore-forming cytolysins (Bishr et al., 2014; Jiang et al., 2010; Ryan et al., 2009; St Jean et al., 2014). These molecules are natively expressed and secreted by bacteria and able to induce apoptosis in mammalian cells (Stanley et al., 1996; Swofford et al., 2014). Unfortunately they represent an special situation, as most macromolecules are not able to pass through bacterial membranes for their release. Fusion with an N-terminal signal peptide to enable bacterial secretion of foreign polypeptides represents a different alternative (Mergulhão et al., 2005; Ni and Chen, 2009), albeit this approach is limited to a small fraction of proteins that are compatible with transport across the bacterial membranes. A broader spectrum of macromolecules can be reach by induction of bacterial lysis, which releases all the bacterial protein content in the tumor environment (Din et al., 2016). For instance, the mitochondria target domain (MTD) of Noxa, implicated in the mitochondrial apoptotic pathway, was delivered fused to a cell penetrated peptide by an attenuated Salmonella strain lysed by the induction of a bacteriophage lysin (Bereswill et al., 2014). Also, syncronized cycles of bacterial lysis, by the induction of bacteriophage lysin under the control of quorum sensing promoters (see later), have been used to release HlyE (Din et al., 2016).

Bacteria can also be modified to deliver nucleid acids such shRNAs or DNA vectors. To achieve this, *E. coli* was transformed with a plasmid encoding the invasin (inv) from *Yersinia pseudotuberculosis* and and listeriolysin O (LLO; hlyA gene) from *Listeria monocytogenes* (Fajac et al., 2004; Grillot-Courvalin et al., 1998). Invasin triggers internalization of the bacteria into a phagosome inside the tumoral cell and LLO appears to facilitate the release of the nucleic acids into the tumor cell cytoplasm (Critchley et al., 2004; Xiang et al., 2006).

In addition, pathogenic bacteria often encodes protein delivery systems that function during infection (Costa et al., 2015) and which may have the potential to be engineered for extracellular and/or intracellular release of therapeutic proteins. Among them, research in our laboratory has used type I secretion systems (T1SS) for extracellular release of antibody fragments (Blanco-Toribio et al., 2010); (Van Impe et al., 2013) and type III secretion systems (T3SS) for intracellular delivery of antibody fragments into the cytoplasm of mammalian tumor cells (Ruano-Gallego et al., 2015).

Interestingly, our group has generated a non-pathogenic E. coli K-12 strain, called SIEC for Synthetic Injector E. coli, able to assemble functional filamentous injectisomes of the T3SS from enterophatogenic *E. coli* (EPEC) for protein delivery into mammalian cells (Ruano-Gallego et al., 2015). However, these systems have not been yet used for protein delivery in tumors in vivo.

2.4 Controlling the expression in vivo of therapeutic proteins in bacteria.

Gene expression add an important control point to bacterial therapies managing the time and location of the drug production. The incorporation of specific promoters sequences uptream of genes encoding for anti-cancer molecules enables the control of the transcription by external signals and minimize toxicity. Some gene products require tighter control due to their cytotoxic effects while non-toxic proteins (e.g. enzymes, antigen) may not need such level of control (Forbes, 2010).

Bacterial promoters used in vivo for controlling therapeutic proteins can be divided in two main cathegories, namely those responding to external inductors and those responding to local enviromental signals (Chien et al., 2017). In *E. coli* and *Salmonella* the promoters that are more commonly used in vivo with external non-toxic inductors are the tetracycline promoter (tetR-PtetA) (Bertram and Hillen, 2008), which can be induced with anhydrotetracycline (aTc), a non-antibiotic tetracycline analog, and the arabinose promoter (araC-PBAD) (Hong et al., 2014), which is induced with L-arabinose, a pentose commongly found in plant polysaccharides and that is metabolized by many bacteria but not by human cells (Jiang et al., 2010; Loessner et al., 2007). An interesting alternative is the salicylate promoter cascade system which amplifies gene expression and is induced with salicylate and its derivatives (e.g. acetyl-salicylate) (Cebolla et al., 2002).

Promoters responding to a physical inducer, rather than a molecular inducer, have also been used in vivo. For instance, γ-irradiation promotes DNA damage, which then causes the degradation of LexA repressor, inducing SOS repair system (Ganai et al., 2009). Thus, use of a SOS-responsive promoter (e.g. recA) can trigger expression of the therapeutic protein of interest upon γ-irradiation (Murphy et al., 2017). Irradiation has a major advantage over the molecular triggers because γ-irradiation can directly penetrate tumor tissue and is not restricted by diffusion limitations (Chien et al., 2017).

On other hand, local enviromental signals can be detected by bacteria to induce the expression of the protein of interest. For instance, since hypoxia is a common condition

present in the core of solid tumors, endogenous *E. coli* promoters controlled by the oxygen-responsive transcriptional regulator FNR can be used for the expression of a therapeutic molecule (Mengesha et al., 2006; Ryan et al., 2009). Also a a synthetic promoter has been constructed for for detect hypoxia in Deyneko et al. by incorporating hypoxia sensing elements (Deyneko et al., 2016).

Interestingly, some promoters activated preferentially within tumors have been identified in Salmonella typhimurium carrying promoter libraries driving expression of GFP (Arrach et al., 2008; Leschner et al., 2012).

In addition, since bacteria grow in high numbers within the tumor tissue and not in healthy tissue, the quorum sensing molecules accumulated in the tumor environment have been used to specifically induce the expression of therapeutic proteins in solid tumors (Din et al., 2016; Swofford et al., 2015).

2.5 Bacterial minicells and tumor therapy.

An interesting alternative to live bacteria could be the use of non-living bacterial minicells (MacDiarmid and Brahmbhatt, 2011). Minicells are small spherical bacteria-derived nanoparticles (ca. 400 nm in diameter) that lack chromosomal DNA and therefore are not able to proliferate. Minicells were originally reported in *E. coli* more than 50 years ago (Adler et al., 1967) but many Gram-positive and Gram-negative bacteria are known to generate minicells by deletion of their minCD genes, which are required for of the proper placement of the cell division FtsZ ring (Romberg and Levin, 2003). Subcellullar location of the Z ring is regulated by the Min proteins and a negative spatial regulator of Z-rings called “nucleoid occlusion” which disable the Z-ring assembly in the space occupied by the bacterial chromosome (Rowlett and Margolin, 2015). In the Δ*minCD* mutants the Z-ring can assemble near the cell poles, resulting in an asymmetrical bacterial division producing a large cell keeping the bacterial chromosomes and a smaller minicell with exactly the same composition of the replicative bacterial cell except for the lack of the chromosomal DNA. Hence, minicells contain cellular membranes, peptidoglycan, ribosomes, RNAs, proteins and plasmids, and they can continue cell processes like ATP synthesis, replication and transcription of plasmid DNA and translation of mRNA (Farley et al., 2016).

The use of minicells as nanovehicles for the delivery of chemotherapeutics (e.g. Doxorubicin) and siRNAs to tumors was originally reported using *Salmonella* minicells (MacDiarmid et al., 2009; MacDiarmid et al., 2007). The accumulation of minicells in solid tumors is only partially due to their small size and was enhanced with bi-specific

antibodies that recognizes the LPS in the minicell surface and a surface receptor on the tumor cell, such as the growth factor receptors EGFR and HER2. After their systemic administration, minicells loaded in vitro with various chemotherapeutics and siRNA were shown to accumulate in solid tumors and release their toxic payload by a

non-completely understood mechanism, which may require lysis of minicells and/or their internalization by tumor cells (reviewed in MacDiarmid, 2011 #284). More recently, different studies have investigated and expanded the use of Salmonella and *E. coli* minicells against various human tumors (Zhang, 2018; Alfaleh 2017; McDiarmid 2016).

Figure 3 Electron micrograph showing an *E. coli* cell producing a minicell (Magnification X66000). Image taken from (Adler et al., 1967).

3. E. coli as a suitable host for synthetic biology engineering and tumor therapies.

Synthetic biology (the deliberate redesign and construction of novel biological parts, devices and systems to perform new functions for useful purposes) provides new opportunities to improve bacterial tumor therapies, designing the whole bacteria, eliminating unwanted genes in the chassis by genome manipulation, and incorporating gene circuits responding to external signals that could control the expression of therapeutic proteins and elicit bacterial biocontainment (Cameron et al., 2014; Chien et

al., 2017; Lee et al., 2018). Given its genetic amenability, *E. coli* is frequently used as a model microorganism in synthetic biology developments. Interestingly, many *E. coli* strains fullfill the properties found in *Salmonella* for good tumor colonization, including its facultative anaerobic growth, its flagellated motility and genetic amenability, plus having the extra benefit of being non-pathogenic and non-invasive. Different studies have demonstrated that non-pathogenic commensal E. coli strains, like the laboratory K-12 strain MG1655 and the probiotic strains (e.g. Nissle 1917) are able to colonize solid tumors grafted in both immunodeficient (athymic Nude) and immunocompetent mouse strains (Balb/c, C57BL/6) (Brader et al., 2008; Kocijancic et al., 2016; Stritzker et al., 2007; Weibel et al., 2008). These studies administered a dose of these natural E. coli strains of ca. $5x10^6$ - $1x10^7$ CFU administered intravenously (i.v.) per animal to obtain efficient tumor colonization (≥ 90%). One hour after systemic administration, approximately 1 x 10^3 CFU reached the tumor, followed by an exponential growth, E. coli reached ca. ~$1x10^8$ CFU/g of tumor in 48 hours and persisted in both immunodeficient and immunocompetent mice over two weeks (Weibel et al., 2008). Most *E. coli* bacteria in tumors were located in the area between the necrotic and the proliferating tumor tissue. Importantly, *E. coli* counts remain low in healthy organs, such as livers and spleens (<$1x10^2$ CFU/g), which contrasts with the reported proliferation in these organs of Salmonella strains.

3.1 Improving E. coli tumor targeting and colonization with programmed bacterial adhesion.

Research in the host laboratory of this PhD thesis is currently aimed to the development of synthetic bacteria based on the modification of *E. coli* chassis from non-pathogenic strains (e.g. K-12, Nissle) with various "genetic modules" that can provide new functional capacities to the resulting bacteria useful for biomedical applications, including bacterial cancer therapies (Pinero-Lambea et al., 2015b). A genetic module that was developed is inteded to program the adhesion properties of the synthetic bacterium, allowing attachment to specific target surfaces or cells (Pinero-Lambea et al., 2015a). This adhesion module is based on the expression of recombinant protein fusions on the surface of bacteria named as "synthetic adhesins" (SAs). SAs comprises the N-terminal fragment of intimin enterohemorrhagic *E. coli* (EHEC), called Neae (Bodelon et al., 2009), which anchors the polypeptide to the bacterial outer membrane with a ß-barrel (Fairman et al., 2012), fused to a single-domain antibody domain, known as nanobody (Nb) review (Muyldermans, 2013), which is displayed on the bacterial surface providing the binding specificity (Pinero-Lambea et al., 2015a). A Nb is recombinant antibody fragment encoded by a single variable gene

segment (VHH) from heavy chain-only antibodies (HCAbs) that are naturally found in camelids (e.tg. dromedaries, llamas) (Muyldermans, 2013). Despite their small size (ca. 15 kDa) and simpler structure than conventional antibodies with heavy and light chains (ca. 150 kDa for IgGs), Nbs able to bind their cognate antigen with high affinity and specificity. In addition, Nbs have very interesting characteristics, such as good expression levels, strict monomeric behaviour, high stability to denaturants and proteases, reversible folding, binding of epitopes distinct from those recognized by conventional antibodies, and show high sequence similarity to human VH3 sequences (Harmsen and De Haard, 2007; Muyldermans, 2013). These properties have made Nbs attractive for various applications, including cancer diagnostics and therapy (Arezumand et al., 2017; Pleiner et al., 2015; Steeland et al., 2016).

The expression of SA gene fusions is driven by a strong constitutive promoter (P_{N25}) (Brunner and Bujard, 1987) and this genetic module was inserted in the chromosome of E. coli replacing the flu gene (Piñero-Lambea et al., 2015) which encodes the Antigen 43 autotransporter (van der Woude and Henderson, 2008). SAs were integrated in E. coli K-12 MG1655 strain lacking type 1 fimbriae (DfimA-H), which is a natural adhesin binding human urothelium (Bower et al., 2005), and carrying a bioluminiscence reporter (luxCDABE operon) replacing another major conserved *E. coli* fimbrial adhesin (mat) (Garnett et al., 2012). A marker-less gene deletion/integration strategy was employed, which leaves no antibiotic resitance genes nor recombination sequences ("scars") in the chromosome of the engineered strain (Posfai et al., 1999). The first SAs generated as a proof-of concept used Nb domains binding model antigens, such as GFP or the translocated intimin receptor (Tir) of enterohemorraghic *E. coli* (EHEC). It was demonstrated that engineered *E. coli* bacteria carrying SAs attached quickly (< 10 minutes) and in high numbers to HeLa tumor cells stably transfected to express on their surface these model antigens (e.g. GFP or TirM fused to a transmembrane domain of platelet-derived growth factor receptor, PDGFR) (Pinero-Lambea et al., 2015a). Importantly, using subcutaneous tumor xenograft mouse models in athymic Nude mice, it was also shown that the engineered *E. coli* bacteria expressing the SA against GFP were able to colonize more efficiently solid tumors of HeLa cells expressing GFP on their surface. The engineered strain colonized >90% of HeLa-GFP tumors in mice using two-order of magnitud lower dose of systemic bacteria (10^5 CFU/mouse) than control strains, which requiered ca. 10^7 CFU/mouse to achieve similar colonization levels (Pinero-Lambea et al., 2015a). The engineered *E. coli* strain proliferated in tumors reaching ca. 10^8 CFU/gr of tumor 4-days after its systemic administration, similar to what was previously reported with wild-

type E. coli K-12 MG1655 (Weibel et al., 2008). In addition, the engineered strains were cleared faster than E. coli K-12 MG1655 from non-target healthy organs (e.g. liver and spleen) probably due to the deletion of natural adhesins (i.e. type 1 fimbriae, Antigen 43 and Mat fimbriae).

Hence, these results with model antigens clearly indicated that SAs allow to program the adhesion capacities of bacteria to target cells, which help to reduce the bacterial dose required to colonize solid tumors expressing the target antigen. Lowering the bacterial dose administered will help to minimize major side effects caused by infection, as those observed in clinical trials with *Salmonella* strains (Nemunaitis et al., 2003; Toso et al., 2002).

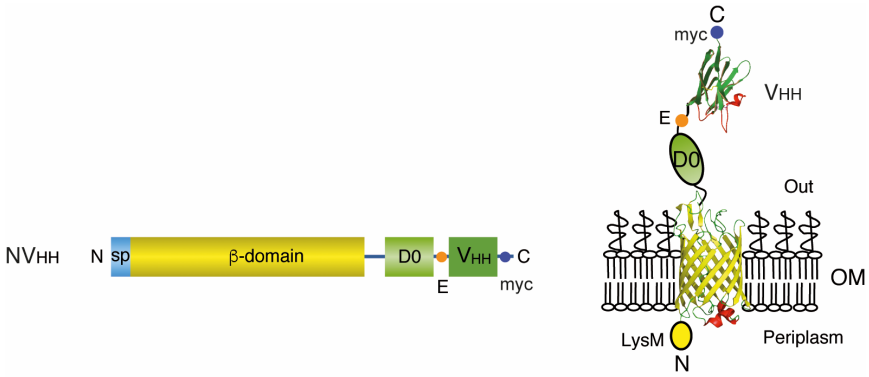


Figure 4. Scheme of the primary structure of SAs (left) showing the N-terminal domain of Intimin (Neae), comprising the signal peptide (SP), LysM, β-domain and D0 Ig-like domain, fused to a variable Ig domain from heavy-chain-only antibodies (VHH). The E-tag and myc-tag epitopes flanking the VHH domain are also indicated. Structural model of a SA fusion protein (right) in the bacterial outer membrane (OM) showing the LysM domain in the periplasm (yellow sphere), the β-barrel of Intimin embedded in the OM, and the D0 and VHH Ig-domains exposed to the extracellular milieu

3.2 Targeting actual tumor-associated cell surface antigens.

An important limitation of the poof-of-concept experiments with SAs is that the targeted proteins (i.e. GFP, Tir) were not actual antigens expressed by tumor cells. Therefore, the model used HeLa tumor cells stably transfected to express these antigens on the cell surface (Pinero-Lambea et al., 2015a). Transfection produced very high expression levels of the model antigen on the tumor cell surface, which are not so common in actual tumor-associated cell surface antigens in vivo. At this point, the next step was to move the SAs to target proteins differentially expressed on tumor cell surface in comparison with the healthy tissue. Expression of cell surface proteins in cancer cell is frequently dysregulated, leading to the production of tumor-associated cell surface antigens that often correlate with tumor progression and metastasis, being an important factor to the prognosis an selection of therapy.

In many different cancer types, especially those of epidermal origin, the ErbB pathway becomes hyperactivated by different mechanisms, including overproduction of ligands, overproduction of receptors or constitutive activation of the tyrosine kinase activity of these receptors (Holbro et al., 2003; Yarden, 2001). The epidermal growth factor receptor (EGFR), belonging to the ErbB family, has a main role in proliferation, cell survival and angiogenesis, and the over-expression of the receptor confers advantages to tumor cells at different stages of tumor development. EGFR is frequently overexpressed in a large number of epithelial tumors, including carcinomas of head and neck, breast, colon, lung, prostate, kidney, ovary, brain pancreas and bladder (Holbro et al., 2003) and currently EGFR the target of many FDA approved drugs (Tebbutt et al., 2013). Cetuximab and Panitumumab are two mAb introduced into clinical practice for treatment of metastatic colorectal and head and neck cancer, both of them target the external part of the EGFR molecule and inhibit the EGFR-generated signaling (Martinelli et al., 2009).

The epidermal growth factor receptor (EGFR) is a single-chain transmembrane protein comprised of an extracellular EGF-binding domain, a short transmembrane sequence, and a cytoplasmic region containing a protein tyrosine kinase domain and a C-terminal phosphorylation domain. Growth factor binding to the extracellular domain of the EGFR (eEGFR) promotes dimerization of the receptor and enhances its intrinsic protein tyrosine kinase activity toward intracellular substrates (Holbro et al., 2003).

In a healthy epithelium the ErbB's receptors have a basolateral location mediating signalling between the mesenchyme and the epithelium for cell growth (Yarden, 2001). During the development of a tumor, epithelial layers become disorganized and cells overexpressing the receptor can be found in the entire tumor volume Figure 2 (Duan et al., 2016; Nguyen and Kattan, 2012; Rebouissou et al., 2014).

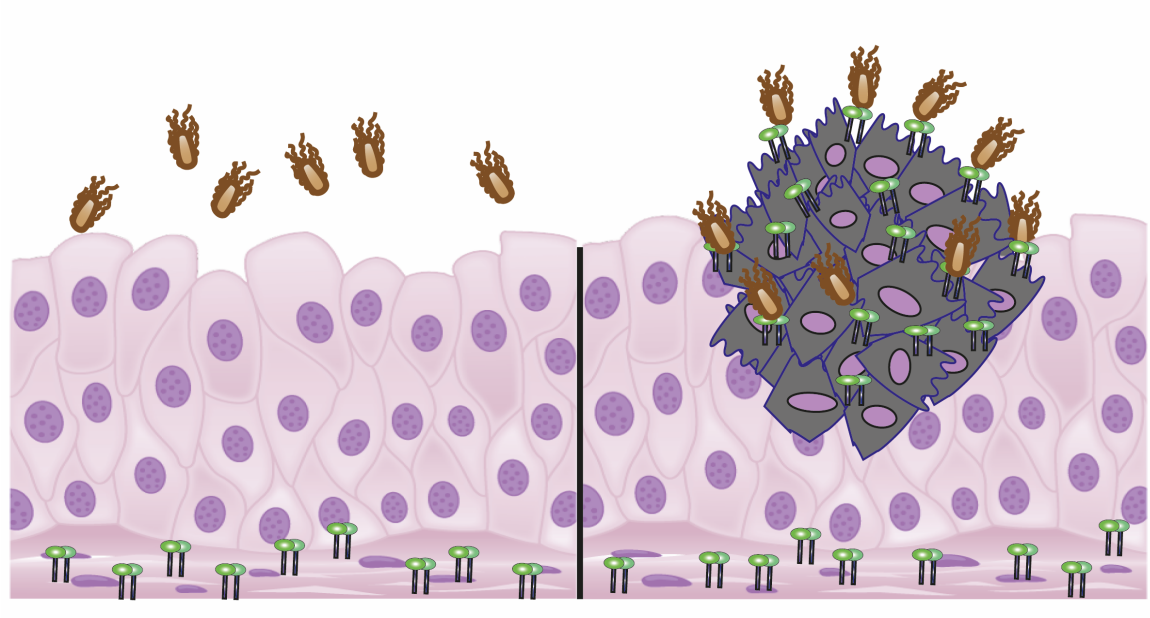


Figure 5. EGFR distribution in a healthy epithelia (left) in comparation with an epithelia containing a tumor (right).

These characteristics make EGFR a perfect candidate for targeting bacteria with SAs against EGFR (Gan et al., 2007). In parallel with the development of SAs, the host laboratory of the PhD thesis demonstrated that the display of Nbs on *E. coli* cell surface with the intimin Neae domain can be also applied for the selection of high-affinity Nb clones binding an antigen of interest from libraries of VHHs obtained after immunization of camelids with these antigens (Salema et al., 2013a). Lymphocytes are isolated from blood samples from the immunized animals and the VHHs are amplified by RT PCR for their cloning fused to intimin Neae domain in a plasmid vector (pNeae2) and electroporated to *E. coli* to generate an immune library with ca. 10e7 independent clones. The *E. coli* display library of Nbs is then incubated with the labelled protein antigen (e.g. by biotinylation) and antigen-binding clones selected by magnetic cell sorting (MACS) and/or fluorescence-assisted cell sorting (FACS) (Salema and Fernandez, 2017). This E. coli display technology was applied to select Nbs binding eEGFR (Salema et al., 2016). To this end, VHHs obtained by immunization of two llamas with human tumor cell line A431 overexpressing EGFR (Gan et al., 2007) was displayed on E. coli with Neae (the final configuration of SAs) and screened for EGFR binders using MACS, with biotinylated extracellular region of EGFR fused to human Fc (eEGFR-Fc) to capture clones. After selection E. coli clones binding specifically eEGFR-Fc by flow cytometry were selected and the sequence of the clones VHHs reveal that five different Nbs were selected. These clones were purified and test in vitro to confirm their ability to bind EGFR-Fc and EGFR-expressing cells (Salema et al., 2016). These Nb clones were then further characterized as part of this PhD thesis in

order to develop bacteria and bacterial minicells targeting tumor cells expressing EGFR.

Material and Methods

4. 1. Bacterial strains and growth conditions.

The E. coli strains used in the experiments described in this work are listed in Table M1. Bacteria were grown in Luria-Bertani (LB) liquid medium and agar-plates (1.5% w/v), at 37°C, unless otherwise indicated. When needed for plasmid or strain selection, antibiotics were added to the media at the following concentrations: chloramphenicol (Cm) at 30 µg/ml, Kanamycin (Km) at 50 µg/ml and ampicillin (Amp) at 150 µg/ml. E. coli DH10B-T1^R strain was used as host for cloning and propagation of plasmids with a pBR origin of replication (e.g. pNV-derivatives). For cloning and propagation of suicide pGE-plasmid derivatives, containing the conditional pi-dependent R6K origin of replication (Stalker et al., 1982), the *E. coli* strains BW25141 or CC118-λpir were used. For inducible expression of the SAs from plasmid vectors with plac promoter (i.e. pNVgfp, pNVegfr), EcM1 bacteria bearing the corresponding plasmid, were grown in LB-Cm at 30°C and 0.05 mM isopropyl-thio-β-D-galactoside (IPTG) was added at an optical density 600 nm (OD_{600}) of 0.5. The cultures were further grown for 2 h at 30°C with agitation (160 rpm). Bacteria with constitutive expression of the synthetic adhesins and the lux operon from the chromosome were always grown statically at 37°C in LB, except for minicells-producer bacterial strains that were also grown at 37°C with agitation (160 rpm). The number of bacterial generations per day was determined according to the formula “Number of generations = (Log Final CFU – Log Initial CFU) / Log 2”.

5. 2. E. coli genome modification and strains construction.

Site-specific deletions and insertions in the chromosome of E. coli were done with the marker-less strategy of genome edition based on expression of I-SceI endonuclease (Posfai et al., 1999; Posfai et al., 2006). Briefly, the E. coli strain to be modified was initially transformed with plasmid pACBSR (Cm^R) (Herring et al., 2003), expressing I-SceI and λ Red proteins under the control of P_{BAD} promoter (inducible with L-arabinose), and subsequently electroporated with the corresponding pGE-based suicide vector (Km^R). Cointegrants were selected on LB-Cm-Km plates incubated at 37°C. Individual colonies were isolated and grown for 6 h in LB-Cm liquid medium containing L-arabinose 0.4% (w/v) with agitation (160 rpm). After this period, a ~1 µl sample of these cultures was streaked on LB-Cm plates using an inoculating loop and incubated overnight. Individual colonies were replicated in LB-Cm and LB-Cm-Km

plates to screen for Km-sensitive colonies that have performed resolution of the co-integrant vector after I-SceI induction. Individual Km-sensitive colonies were screened by PCR with specific oligonucleotides (Table M3) to identify those with the desired modification in their chromosome (i.e. deletion, insertion, substitution). Plasmid pACBSR was cured from the final strains by growth in liquid LB and streaking on LB-plates. Individual colonies were replicated in LB and LB-Cm plates to screen for Cm-sensitive colonies.

The change of specificity of a SA already integrated in the *E. coli* chromosome was performed by the substitution of the variable part of the SA using the suicide vector denominated pGErecomb that contain the new VHH flanked by the homology region 1 corresponding to the intimin part from the SA structure and the HR 2 from the flu gene where is integrated the SA. For the integration we used the same protocol than before, in the Figure 6 we generate a SA against EGFR by substitution of the Vgfp in the synthetic adhesin already integrated in the target strain for the Vegfr clonated in the plasmid. Construction details of individual E. coli strains are described in Table M1.

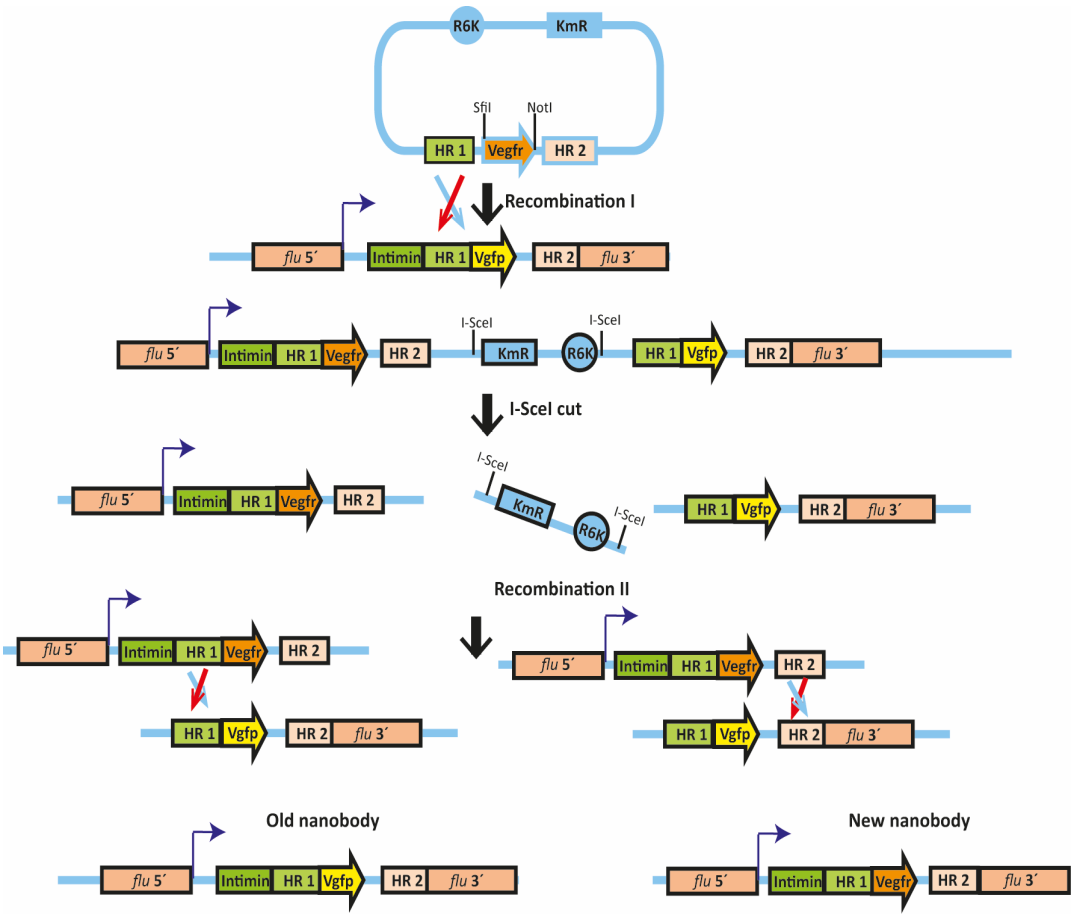


Figure 6. Site-specific change of specificity of the synthetic adhesin in the chromosome of *E. coli.* Scheme showing the integration of the vhh against egfr in substitution of the vgfp from the

synthetic adhesin express in E .coli. Integration is performed using the pGE Recomb flu-SA::Vegfr suicide plasmid containing the π dependent R6K origin of replication, a km resistance gene (KmR) and the Vegfr gene flanked by HRs, corresponding to 3´´-ends of the eae sequence from the SA and the flu gene, and two I-SceI restriction sites. Homologous recombination of the suicide plasmid with the chromosome (Recombination I) leads to KmR – cointegrants that are later resolved by the expression of SceI endonuclease from the helper plasmid pACBSR (Table M2). The double strand breaks, generated by cleavage of DNA with I-SceI are repaired by a second homologous recombination (RecombinationII) that could either, revert the cointegrant to the wild type situation, or lead to chromosomal integration of the Vegfr replacing the original Vgfp.

Table M1. Bacterial strains used in this thesis.

Name	Genotype	Reference
DH10B-T1^R	*F- λ-) mcrA Δmrr-hsdRMS-mcrBC φ80lacZDM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK rpsL (Str^R) nupG tonA*	Novagen
BW25141	*(F- λ-) Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), Δ(phoB-phoR)580, galU95, ΔuidA3::pir, recA1, endA9(del-ins)::FRT, rph-1, Δ(rhaD-rhaB)568, hsdR5(F- λ-) Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), Δ(phoB-phoR)580, galU95, ΔuidA3::pir, recA1, endA9(del-ins)::FRT, rph-1, Δ(rhaD-rhaB)568, hsdR51(F- λ-) Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), Δ(phoB-phoR)580, galU95, ΔuidA3::pir, recA1, endA9(del-ins)::FRT, rph-1, Δ(rhaD-rhaB)568, hsdR51*	Datsenko and Wanner, 2000*)*
CC118 λpir	*Δ(ara-leu) araD ΔlacX74 galE galK phoA20 thi-rpsE rpoB argE(Am) recA1, λpir*	Herrero et al., 1990)
MG1655	*K-12 (F- λ-)*	
EcM1	*MG1655ΔfimA-H*	Salema et al., 2013)
EcM1lux SAgfp	*EcM1Δflu::P_{N25}-SAgfp ΔmatB::P_2-luxCDABE*	Carlos Piñero thesis
EcM1lux SAtir	*EcM1Δflu::P_{N25}-SAtir ΔmatB::P_2-luxCDABE*	This work
EcM1lux SAegfr1	*EcM1Δflu::P_{N25}-SAegfr1 ΔmatB::P_2-luxCDABE*	This work
EcM1lux SAegfr2	*EcM1Δflu::P_{N25}-SAegfr2 ΔmatB::P_2-luxCDABE*	This work
EcM1 ΔfliSAegfr		Carlos Piñero
EcM1luxSAegfrΔmotA	*EcM1Δflu::P_{N25}-SAega ΔmatB::P_2-luxCDABE ΔyeeJ:: Ptac-gfp ΔmotA*	This work
EcM1luxSAegfrΔflhDC	*EcM1Δflu::P_{N25}-SAega ΔmatB::P_2-luxCDABE ΔyeeJ:: Ptac-gfp ΔflhDC*	This work
EcM1luxSAegfrΔcheY	*EcM1Δflu::P_{N25}-SAega ΔmatB::P_2-luxCDABE ΔyeeJ:: Ptac-gfp ΔcheY*	This work
EcM1luxSAegaΔcheZ	*EcM1Δflu::P_{N25}-SAega ΔmatB::P_2-luxCDABE ΔyeeJ:: Ptac-gfp ΔcheZ*	This work
EcM1luxSAegaΔnlpE	*EcM1Δflu::P_{N25}-SAega ΔmatB::P_2-luxCDABE ΔyeeJ:: Ptac-gfp ΔnlpE*	This work
EcM1luxSAegaΔdgcZ	*EcM1Δflu::P_{N25}-SAega ΔmatB::P_2-luxCDABE ΔyeeJ:: Ptac-gfp ΔdgcZ*	This work
EcM1luxSAegaΔycgR	*EcM1Δflu::P_{N25}-SAega ΔmatB::P_2-luxCDABE ΔyeeJ:: Ptac-gfp ΔycgR*	This work
EcMini-WT	*MG1655 ΔminCD*	Carlos Piñero thesis
EcMini-SAegfr	*EcM1Δfli ΔmsbB ΔminCD Δflu::P_{N25}-SAega ΔyeeJ::Ptac-gfp*	This work

EcMini-SAtir	*EcM1Δfli ΔmsbB ΔminCD Δflu::P_{N25}-SAtir ΔyeeJ::Ptac-gfp*	This work
EcMini-SAegfr2	*EcM1Δfli ΔmsbB ΔminCD Δflu::P_{N25}-SAegfr2 ΔyeeJ::Ptac-gfp*	This work
EcM1lux SAegfr2 Inva-gfp	*EcM1Δfli ΔmsbB ΔminCD Δflu::P_{N25}-SAegfr2 ΔyfaL::AraCP_{BAD}invasin*	This work
EcM1lux SAegfr2Inva-llo-gfp	*EcM1Δfli ΔmsbB ΔminCD Δflu::P_{N25}-SAegfr2 ΔyfaL::AraCP_{BAD}invasin Δyra::P_{BAD}llo*	This work
EcM1lux SAegfr2Inva-pelBllo-gfp	*EcM1Δfli ΔmsbB ΔminCD Δflu::P_{N25}-SAegfr2 ΔyfaL::AraCP_{BAD}invasin Δyra::P_{BAD}pelBllo*	This work
EcM1lux SAegfr2Inva-llo-gfpΔdapA	*EcM1Δfli ΔmsbB ΔminCD Δflu::P_{N25}-SAegfr2 ΔyfaL::AraCP_{BAD}invasin Δyra::P_{BAD}llo ΔdapA*	This work
Salmonella WT	*Salmonella enterica sv. typhimurium*	Garcia del Portillo lab
Salmonella ΔflhDC	*Salmonella enterica sv. Typhimurium :: ΔflhDC*	Garcia del Portillo lab

Table M2. Plasmids used in this thesis.

Name	**Relevant properties**	**References**
pNVgfp	*pNeae2-derivative:VHH anti-GFP fused to Neae[Intimin$_{EHEC}$(1-659)-E-Vgfp-myc-tag]*	(Salema et al., 2013b)
pNVegfr$_n$	*pNeae2-derivative:VHH anti-EGFR$_n$ fused to Neae[Intimin$_{EHEC}$(1-659)-E-Vgfp-myc-tag]*	(Salema et al., 2016)
pGErecombVtir	*pGE derivative: changing the specificity of a SA for the Vtir nanobody*	This Work
pGErecombVegfr1	*pGE derivative: changing the specificity of a SA for the Vegfr1 nanobody*	This Work
pGErecombVegfr2	*pGE derivative: changing the specificity of a SA for the Vegfr2 nanobody*	This Work
pACBSR	*(Cm^R), p15A-ori, P_{BAD} promoter, I-SceI endonuclease, λRed*	
pGEΔypjA::TetRPtetgfp	*pGE derivative: integration of the gfp in substitution of the ypjA E. coli K-12 gene under TetR-Ptet control.*	This Work
pGEΔyfaL::AraCP_{BAD}invasin	*pGE derivative: integration of the invasin in substitution of the yfaL gene from E. coli K-12 under control of AraC-P_{BAD}.*	This Work
pGEΔyra::P_{BAD}llo	*pGE derivative: integration of the llo in substitution of the yra E. coli K-12 gene under P_{BAD} control.*	This Work
pGEΔyra::P_{BAD}pelB-llo	*pGE derivative: integration of the llo in substitution of the yra E. coli K-12 gene under P_{BAD} control.*	This Work
pGEΔdapA	*pGE derivative: deletion of the dapA gene from E. coli K-12*	This Work
pGEΔmotA	*pGE derivative: deletion of the motA gene from E. coli K-12*	This Work
pGEΔflhDC	*pGE derivative: deletion of the flhDC gene from E. coli K-12*	This Work
pGEΔfliCD	*pGE derivative: deletion of the fliCD gene from E. coli K-12*	This Work
pGEΔcheY	*pGE derivative: deletion of the cheY gene from E. coli K-12*	This Work
pGEΔcheZ	*pGE derivative: deletion of the cheZ gene from E. coli K-12*	This Work

pGEΔnlpE	*pGE derivative: deletion of the nlpE gene from E. coli K-12*	This Work
pGEΔdgcZ	*pGE derivative: deletion of the dgcZgene from E.coli K-12*	This Work
pGEΔycgR	*pGE derivative: deletion of the ycgR gene from E.coli K-12*	This Work
pRI203	*Encoding the invasion from Y.pseudotuberculosis*	(Isberg and Falkow, 1985)
pSH2	*Encoding the fimH operon form UPEC strain J96*	(Song et al., 2007)
pBAD18ßla	*Encoding ß-lactamase gene under control of the $AraCP_{BAD}$ promoter*	David Ruano thesis
pBAD18T3SS-V_{gfp}ßlaCesT	*Encoding ß-lactamase gene under control of the $AraCP_{BAD}$ promoter with the secretion signal for injection with the typeIII secretion system.*	David Ruano thesis

Table M3. Primer list used in this thesis.

Name	***Sequence***
eae 4	CGTAATGGCAATAGCTCTAACAATGTA
Km seq reverse	GCAAACTGATTGGCTGCATTGATG
ypjA HR5 XhoI	CTGGCCCAATCTATATTTGCCCAAC
ypjA HR5 SacI	GTAGTGAAGGCAGCCATCCCGG
ypjA HR3 SpeI	GTATGGAAGTTGAGCCGTGGCTG
ypjA HR3 SphI	GCTGGTATCACTCTGGCCGATGC
Fw AraC	CGGAGATCTTTATGACAACTTGACG
Rv PBAD	GACTCTAGACTCGCAAAAAAACGGG
Check PBAD Rev	AGTTAGGGATTAGCGTCTTAAGCTG
Check PBAD Fw	CTATGCCATAGCATTTTTATCCATA
Invasina RBST7 SalI Fw	GAGTCTAGAGTCGACAAGAAGGAGATATACATATGATGGTTTTCCAGCC AATCAG
Invasin BamHI Rv	GGATCCTTATATTGACAGCGCACAG
LLO RBS B0030 NotI Fw	GGACTAGTGCGGCCGCATTAAAGAGGAGAAATACTAGATGGATGCATC TGCATTCAATAAAGAAAATTC
LLO SpeI Rv	GTGAACTAGTATTATTCGATTGGATTATCTACTTTATTACTATATTTCG
motA HR5 Rv SacI	CGGTCTCGAGCTGAAAACCGGTTTGTG
motA HR5 Fw XhoI	GCAGGAGCTCGACATCATCCTTCCACTG
motA HR3 Fw SacI	GCAGGCATGCGTACAGGCGCAATGG
motA HR3 Rv SpeI	CCGTGAGCTCGAAGAATCAAGCGCATCC
Check Fw motA	GGCATGCTGCCATTCTCAACCG
Check Rv motA	GCATTGGCCCGATCGGCAG
Check Fw flhCD	CGCTGGGGCGTCTTATGAGCC
Check Rv flhCD	CAGCCGCAACAATACCAAACG
fliCD HR3 Fw	GGTGCATGCGGCGTCATAGCGTTCGACGG
fliCD HR3 Rv	ACGGAGCTCCCTGATTAACTGAGACTGACGG
fliCD HR5 Fw	AGGGAGCTCCGTTATAAGTAGCCCTCTGTCCAG
fliCD HR5 Rv	GGTCTCGAGGGGTAACAAGAAGGCAACGG
Check Fw fliCD	GCACCGCATTCACATACGCC
Check Rv fliCD	GCGCCACGGATACGCTGCAC
cheY 5 XhoI	CGGTCTCGAGGCAACTGGCGTTCGATCTCCTG
cheY 5 SacI	ACTGGAGCTCGGATGCGACTATGATGCAACCATC
cheY 3 SacI	TCCGCATCCGAGCTCTTCACACTCCTGATTTAAATACGTATC
cheY 3 SpeI	TAGGACTAGTCAGCATATGCCGCCCGGTTTCACCCGCTCT

Check cheY Fw	CGCGTCAGGCTGCCGATGCGC
Check cheY Rv´	CATCAATATGGGTGGTGTCTG
cheZ HR5 Fw XhoI	CGGTCTCGAGAGTCGCATCCTCACATGCCCAGTTTCTC
cheZ HR5 Rv SacI	TGGGAGCTCGTGGTGTCTGCGAAGTGGTCGATC
cheZ HR3 Fw SacI	CCACGAGCTCCCAGCACCACGCCGCTAATC
che Z HR3 Rv SpeI	TAGGACTAGTTTTGTATTGCCTGATGTGGCGTGA
Check cheZ Fw	CGCGTCAGGCTGCCGATGCGC
Check cheZ Rv	CATCAATATGGGTGGTGTCTG
dcgZ HR3´ XhoI	CGGTCTCGAGCGCTGTTTGTCATCTTAGCG
dcgZ HR3´ SacI	GCAGAGCTCGCTCCGTTTAACATTCATTG
dcgZ HR5´ SacI	GAGCGAGCTCTGCCACTCCTTTTTCACAG
dcgZ HR5´ SpeI	AGGACTAGTGCTCCTCATGCAATAGAAGATGGC
Check dgcZ Fw	GCTAACATCCGCGCGCTGATGAC
Check dgcZ Rv	GTCATGCCTTATGCGTTGGC
nlpE HR5 Fw XhoI	CGGTCTCGAGGGATGGCGTGATCTGGTTATC
nlpE HR5 SacI Rv	GGGGAGCTCCCATTCCTTCTTTTTATCC
nlpE HR3 SacI Fw	TGGGAGCTCCCCTCTTGAGACAGAAACA
nlpE HR3 SpeI Rv	AGGACTCGTGCTGGTGACTCGCCAGATGCTC
Check nlpE Fw	GACAGCGATGGCTGTAATCAG
Check nlpE Rv	GTCGGCGCACCGGTATCCGGA
dapA HR5 Fw	GGTCTCGAGGAGATTTGATAACGACCACGATACTGCTCG
dapA HR5 RV Fusion	GACACTTGTTTGCACAGAGGATGGCCCAGTTTAGGGAGATTTGATGGCT TACTC
dapA HR3 Fw Fusion	CTAGGACTAGTGCTCGCGCGTCGAAAAGTGCTGTGAAGC
dapA HR3 RV	CTAGGACTAGTGCTCGCGCGTCGAAAAGTGCTGTGAAGC

6. 3. Surface plasmon resonance.

SPR measurements were performed using Biacore 3000 instrument (GE Healthcare). All proteins were dialyzed against HEPES-buffer (20mM HEPES 200mM NaCl (pH 7.4) sterile filtered and degassed) at 4ºC o/n. e-EGFR-Fc was immobilized on CM5 chip (GE Healthcare) by amino-coupling at 1000 response units (RUs). For amino-coupling immobilization EDC/NHS at 5µl/min was injected for 6 min. The eEGFR-Fc at 5µG/ml in 10mM AcNa pH 5.0 was injected for 2 min. Finally the immobilization was blocked by 1M ethanolamine- HCl, pH 8,5 for 6 min. For competition assays eEGFR-Fc was immobilized at 6000 RUs. For this immobilization EDC/NHS at 5µl/min was injected for 7 min and blocked with 1M ethanolamine-HCl, pH 8,5 for 7 min.

For determination of binding-kinetic dilutions of purified vEGFRs from 0.12 nM (vEGFR1), from 0.4 nM to 33 nM (vEGFR2), from 0.14nM to 100nM (vEGFR3), from 0.41 to 100nM (vEGFR4) and from 0.41 nM to 300 nM (vEGFR5) were injected at flow of 30 µl/min and washed with HEPES at the same flow. For competition assays vEGFR1-vEGFR4 were diluted to 100nM while vEGFR5 was used at 400nM and EGF

at 300 nM. The two competitor molecules were injected sequentially at flow of 30 µl/min. Sensorgrams were generated and the chip was regenerated each cycle using three injections (10µl) of 10mM glycine-HCl pH 2.5. For binding-kinetics, sensorgrams with the different concentrations of analyte were overlaid, aligned and analyzed with BIAevaluation 4.1 software (GE Healthcare). All data were processed using double-referencing methods (Myszka, 2000).

7. 4. Flow cytometry analyses.

Bacteria (equivalent to a final OD600 of 1.0) were harvested from cultures by centrifugation (4000 xg, 3 min), washed with PBS and resuspended in 1 ml of PBS containing 10% (v/v) goat serum (Sigma). An aliquot sample of 200 µl was incubated for 1 h on ice with anti-myc mAb clone 9B11 (1:200; Cell Signaling Technology). Next, bacteria were washed in PBS, resuspended in 500 µl of PBS containing 10% (v/v) goat serum, and stained for 40 min in the dark with Alexa 488-conjugated anti-mouse IgGs (1:500; Molecular Probes, Life Technologies). Bacteria were finally washed and resuspended in a final volume of 1 ml of PBS, and analyzed in a flow cytometer (Gallios, Beckman Coulter).

For cell staining 5X104 cells of the studied cell lines in DMEM supplemented with FBS and Glutamine, were seeded onto P6 Thermo Fisher culture plates and grown in an incubator at 37º C and 5% CO2 for 24 hours.

The cells were washed with attemperated DMEM and trypsinized with 1ml of tripsin/EDTA 0,05% (Gibco) for 5 minutes. Next the trypsin was neutralize with DMEM 10%FBS and the cells were washed twice with 1ml of Hank Balance medium 1200 rpms 3 min and resuspended in 500µl of filtered PBS containing the primary antibody anti-EGFR (1µg/ml, Calbiochem) incubated for 1 hour on ice. The cells were washed twice with 1ml of filtered PBS and incubated with the secondary antibody goat anti-mouse Alexa 488 (1:500) in 500 µL volume for an extra hour. Cells were washed twice and resuspended in 300µl of filtered PBS for analysis in the flow cytometer (Gallios Coulter Beckman).

8. 5. Protein extract preparation, SDS-PAGE, and Western blots.

WB of phosphorylated Epidermal Growth Factor Receptor (EGFR) was performed using Her14 mice fibroblast expressing human EGFR or 3T3 2.2 mice fibroblast expressing low levels of mouse EGFR.

Cells were seeded in 6-well tissue culture plates (BD Falcon) (~10^5 cells/well), grown for 24 h at 37°C with 5% CO_2, grown as monolayer in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine (complete DMEM), at 37°C with 5% CO_2. After 24 h we removed the media and add DMEM media supplemented with 2mM glutamine but without fetal bovine serum for 16 hours.

For detecting EGFR phosphorylation the different treatment with EGFR, EGFR + purified Nb anti EGFR or just Nb anti-EGFR were added in DMEM media for 30 minutes.

Two different concentrations of Nb´s was employed 30nM or 100nM, in the case of the EGF just one concentration was used 15nM in all the cases.

After the incubation the cells were washed 3 times with 2ml of Hank balance salt medium (HBSS Sigma) previously chilled at 4ºC.

Cells were incubated 30 min shaking 50 rpm´s at 4ºC with 500µl of lysis buffer also pre-chilled at 4ºC (50mM Tris-HCl pH 7,4, 1mM EDTA, 150mM NaCl, 1%Triton X100, 2mM Ortovanadate (Na3VO4), 10mM NaF, 1mM PMSF, Roche complete inhibitor EDTA-free). The samples were incubated with the buffer for 30 min and removed with the cell scrapper.

The lysis process was complete passing the samples 4 times through a 25 G needle (Microlance 25G 5/8") using a 1ml syringe and incubating in Eppendorf® tubes 10 min on ice.

The tubes were centrifuge for 10 min at 4ºC for 10 min at max speed and recover the supernatant.

Cells extracts were boiled with SDS sample buffer for 15 minutes and charge in a SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting conditions to polyvinylidene difluoride membrane (PVDF, Immobilon-P; Millipore) have been reported (Bodelon et al., 2009; Bodelón et al., 2009; Salema et al., 2013a). For immunodetection, the PVDF-membranes were incubated with anti Anti-EGFR-P clone Y1068 (1:5000; Cell Signaling Technology) to detect the phosphorylated EGFR, and anti-rabbit IgG-peroxidase (POD) conjugate (1:5000; Sigma), and with anti-EGFR clone 528 which recognize human EGFR (1:5000; Cell Signaling Technology), and anti-mouse IgG-peroxidase (POD) conjugate (1:5000; Sigma).

For bacteria and minicells SAs expression detection, whole-cell protein extracts were prepared by mixing 100 µl of a bacterial suspension (OD600 1.0) or minicells

suspension (1,2 x 10^9 minicells/ml) with 100 µl of PBS (phosphate-buffered saline) and 2X sodium dodecyl sulfate (SDS) sample buffer or urea-SDS sample buffer 2X. The SDS sample buffer contains 60 mM Tris-HCl (pH 6.8), 1% (wt/vol) SDS, 5% (vol/vol) glycerol, 0.005% (wt/vol) bromophenol blue, and 1% (vol/vol) 2-ME. The urea-SDS sample buffer contains 60 mM Tris-HCl (pH 6.8), 2% (wt/vol) SDS, 4 M urea, 5 mM EDTA, 5% (vol/vol) glycerol, 0.005% (wt/vol) bromophenol blue, and 1% (vol/vol) 2-ME.

(PAGE) was performed by using the Miniprotean III electrophoresis system (Bio-Rad).

For immunodetection, the PVDF-membranes were incubated with anti-myc mAb clone 9B11 (1:2000; Cell Signaling Technology) to detect myc-tagged SAs and anti-mouse IgG-peroxidase (POD) conjugate (1:5000; Sigma) as secondary antibody. GroEL was detected with anti-GroEL mAb-POD conjugate (1:5000; Sigma) and Omp-A with anti-Omp-A polyclonal serum (1:10000) and anti-rabbit IgG-peroxidase (POD) conjugate (1:5000; Sigma) as secondary antibody. Membranes were developed by measure of chemiluminescence using the Bio-Rad Clarity Western ECL Substrate.

9. 6. In vitro cell culture.

The epithelial tumor cell lines Her14, 3T32.2, T24 (ATCC), UMUC3 (ATCC), were grown as monolayer in Dulbecco's modified Eagle's medium (DMEM Sigma) supplemented with 10% FBS (Gibco) and the RT112 (Sigma) and DLD1 (ATCC) cell lines in RPMI medium (Sigma) with 10% FBS, while HCT116 (ATCC) human colon epithelial tumor cells were grown in Mac Coy´s medium (Sigma) supplemented with 10% fetal bovine serum (FBS Gibco) grown in the incubator at 37°C with 5% CO_2.

Cells were cultured in T-75 Flask from Falcon and subcultured periodically when the cultures reached a confluence of ~80%.

10.7. Bacterial adhesion assays to in vitro cultured cells and immunofluorescence microscopy.

Bacteria from the indicated E. coli strain were harvested by centrifugation (4000 xg, 3 min) from static liquid LB cultures grown overnight at 37 ^oC. An OD_{600} of 1.0 from these cultures grown statically was determined to contain ~3x10^8 CFU/ml by plating in LB-agar. Bacteria were washed in DMEM medium and resuspended at 3x10^7 CFU/ml in DMEM. For an infection at MOI 300:1, a 1 ml sample of this bacterial suspension was added to a single well of a 24-well tissue culture plate having the indicated cell line

(~10^5 cells/well). Cells were grown on sterile coverslips (13 mm diameter, Thermo Fisher) placed at the bottom of the well. After 1 h infection at 37 ^oC, the wells were aspirated and washed five times with 1 ml PBS at room temperature. For the adhesion assays performed with the flagella mutant strains the infection time was reduced to 30 minutes. The coverslips were fixed for 20 min at room temperature with 0. 5 ml of a paraformaldehyde 4% (w/v) solution in PBS and washed three times with 1 ml of PBS. Coverslips were blocked and stained for 1 h at room temperature in a wet chamber with 50 µl of PBS-10% goat serum solution, for Her14, 3T3 2.2 cells immunofluorescences a rabbit polyclonal serum anti-E. coli O and K antigenic serotypes (1:1000; Biodesign) and mouse mAb anti-EGFR (1:500; Calbiochem) was used . The coverslips were washed by immersion 10 times in a large volume of PBS (100ml), placed again into the wet chamber and incubated for 40 min at room temperature with 50 µl of PBS-10% goat serum solution, having a goat anti-rabbit IgG-Alexa 488 conjugated secondary antibody (1:500; Molecular Probes, Life Technologies), anti-mouse IgG-Alexa 594 conjugated secondary antibody (1:500; Molecular Probes, Life Technologies) and DAPI (1:500; Sigma).

For bladder and colon human epithelial tumor cells immunofluorescences, rabbit polyclonal serum anti-E. coli O and K antigenic serotypes (1:1000; Biodesign) was used. The coverslips were washed by immersion 10 times in a large volume of PBS (100ml), placed again into the wet chamber and incubated for 40 min at room temperature with 50 µl of PBS-10% goat serum solution, containing goat anti-rabbit IgG-Alexa 488 conjugated secondary antibody (1:500; Molecular Probes, Life Technologies), Phalloidin Alexa 594 conjugated secondary antibody (1:500; Molecular Probes, Life Technologies) and DAPI (1:500; Sigma).

Next, the coverslips were washed with PBS as above, the excess liquid was removed touching a kimwipe with the edge of the coverslip, and mounted with 5 µl of Prolong (Invitrogen) on glass slides. The samples were examined by confocal microscopy (Leica TCS SP5 multispectral confocal system or Leica TCS SP8 multispectral confocal system).

For live cell video microscospy, Her14 cells, grown on 6-well µ-slide (Ibidi), were infected with 3x10^6 CFU of EcM1luxSAegfr, EcM1luxSAegfr∆cheY or EcM1luxSAegfr∆cheZ in a final volume ~0.3 ml/well (MOI 30:1). Videos were acquired at real time with *Yoda (WF-TIRFM)* microscopy.

8. Bacterial adhesion assays to in vitro cultured cells under flow conditions.

For adhesion assays onto Her14 cells with flagella mutant strains under flow conditions, 8,4 x 10^4 Her14 cells were grown into the Ibidi slide VI$^{0.4}$ channel in 30 µl of media (DMEM supplemented with FBS 10%) for 24 hours. The strains EcM1luxSAegfr, EcM1luxSAegfrΔflhCD or EcM1luxSAegfrΔmotA from an overnight static culture in LB with IPTG for the expression of gfp, were resuspended at 1.0 OD´s/ml and passed through the channel at two different flow conditions, 20µl/min and 100µl/min using a peristaltic pump. All the samples were examined by the epifluorescence microscopy in a Zeiss Axio imager microscopy.

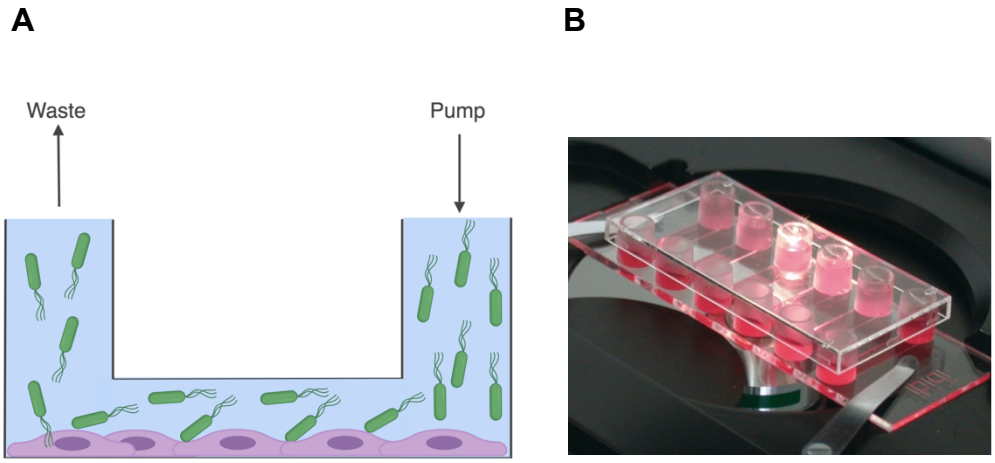


Figure 7. A. Flow device scheme. Cells were seeded into the bottom of an Ibidi slide VI$^{0.4}$ and the bacteria pumped into the channel, **B.** Ibidi slide VI0.4 image placed into the microscope.

11.9. Internalization assay E.coli into human epithelial tumor cells.

EcM1lux SAegfr2Inva-llo-gfp bacterial strains were harvested by centrifugation (4000 x g, 3 min) from static liquid LB cultures grown overnight at 37 ^oC. An OD_{600} of 1.0 from these cultures grown statically was determined to contain ~3x10^8 CFU/ml by plating in LB-agar. Bacteria were washed in PBS and resuspended at 1x10^7 CFU/ml in DMEM with anhidrotetracycline (200 ng/ml) for induction of GFP with the TetRPtet promoter for visualizing the bacteria once it is internalized. For the infections at MOI 30:1, a 1ml sample of this bacterial suspension was added to a single well of a 24-well tissue culture plate having T24 or HCT116 cells (~10^5 cells/well) growing over sterile coverslips (13 mm diameter Thermo Fisher), placed at the bottom of the well 48 h earlier. After 60 minutes of infection at 37 ^oC, the wells were aspirated and washed five times with 1 ml of DMEM at room temperature.

For the invasion of the tumor cells, Arabinose 4% was added to the DMEM media for 4 hours extra, anhidrotetracycline is maintained at the same concentration.

Coverslips were washed five times with 1ml of DMEM each time and fixed in the case of the invasion at 4 hours with 4% of paraformaldehyde for 20 minutes. In the case of the 24 hours measurement, the cells were incubated with 100µg/ml of gentamycin (Gm) for 1,5 hours and overnight with 10 µg/ml of Gm, after this time the samples were fixed as before (see Figure 8).

The coverslips were washed 10 times and placed in a wet chamber and incubated for 40 min at room temperature with 50 µl of PBS-10% goat serum solution, having rabbit polyclonal serum anti-E. coli O and K antigenic serotypes (1:1000; Biodesign), The coverslips were washed by immersion 10 times in a large volume of PBS (100 ml), placed again the wet chamber and incubated for 40 min at room temperature with 50 µl of PBS-10% goat serum solution, having a goat anti-rabbit IgG-Alexa 546 conjugated secondary antibody (1:500; Molecular Probes, Life Technologies), Phalloidin Alexa 660 conjugated secondary antibody (1:500; Molecular Probes, Life Technologies) and DAPI (1:500; Sigma).

After staining, samples were mounted with 5 µl of Prolong (Invitrogen) on glass slides, and examined by confocal microscopy Leica TCS SP5 multispectral confocal system or Leica TCS SP8 multispectral confocal system for the actin polymerization images.

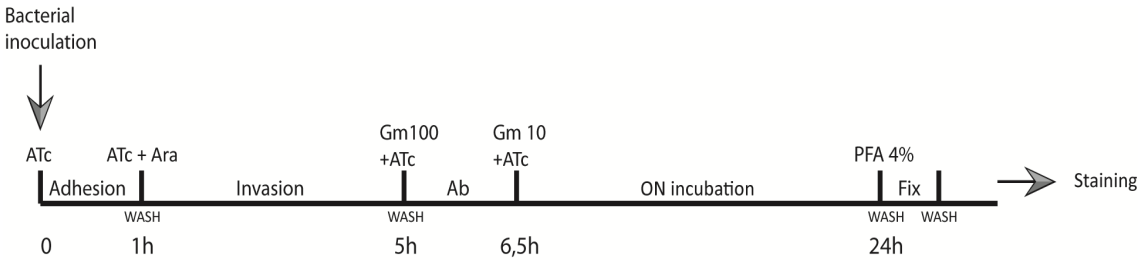


Figure 8. Workflow of the internalization assay.

12.10. LIVE DEAD Cell assay

For the Live Dead cell assay we repeat the same protocol that is described in the Figure 8 and at 24 hours we performed a Live Dead cell staining to check by flow cytometry the percentage of dead cells.

For the staining we recover the supernatant of each well for the cells that were in suspension. The attached cells were trypsinized with 300µl of pre-heat trypsin/EDTA

0,05% (Gibco- 25300054) for 2 min and neutralized with 300µl of DMEM FBS 10% and added to the tube that contains the cells in suspension. The cells were washed twice with 1 ml of filtered PBS 1200rpm 3min and resuspended in filtered PBS containing the dyes. For preparing the dyes we made a 80-fold dilution of calcein AM (Component A) in DMSO to make a 50 µM working solution. The working solution should be used within one day. Dye solution was prepared with 2 µL of 50 µM calcein AM working solution and 4 µL of the 2 mM ethidium homodimer-1 stock that were added to 1 ml of filtered PBS and protected from light.

Each pellet of already washed cells was resuspended in 300µl freshly prepared dye solution and incubated for 30 minutes on ice. The samples were analized using the flow cytometer (Gallios, Beckman Coulter).

11. Apoptosis assay.

For the apoptosis assay we repeated the same protocol but we used Annexin-APC and DAPI staining for differenciation of the apoptotic cells against the dead cells. The cells were washed with PBS twice and resuspended in PBS with Annexin binding buffer 1X at a concentration of 10 million cells/ml. 1µl of Annexin –APC and 1µl of DAPI were added to 100µl of the cell suspension and incubated 10min at room temperature in the dark. After the incubation, 200µl of filtered PBS was added to each sample before their analysis on the flow cytometer (Gallios, Beckman Coulter).

12. ß-lactamase assay.

The human cell line HCT116 was grown in monolayer up to 80% confluence (2 x 10^4cells per well) in DMEM supplemented with 10% of FBS and 2mM glutamine in a P96 well plate Black/Clean tissue cultured treated plate from Falcon (353219). The medium was removed and cells were washed three times with Hank´s Balance salt solution (HBSS Sigma). Next cells were infected with different bacterial strains containing plasmids expressing the ß-lactamase gene under $AraCP_{BAD}$ promoter control: *EcM1luxSAegfr2Inva-llo-gfp, EcM1lux SAegfr2Inva-pelBllo-gfp, EcM1lux SAegfr2Inva-llo-gfpΔdapA* and SIEC. Bacterial strains were added to the culture at 30:1 MOI in 200µl of DMEM without glucose (Gibco) for 30 minutes and washed with pre-heated HBSS five times. We added DMEM without glucose and supplemented with 0.4% of arabinose for the induction of ß-lactamase production and invasion for 4 hours.

After this time, we washed three times each well with 200µl of HBSS and treated the invaded cells with the antibiotic treatment (1,5 hours Gm 100µg/ml and 16 hours Gm 10 µg/ml). At 24 hours post-infection the medium was removed and the cells were washed three times with HBSS. Next 200µl of HBSS and 20 µl of ß lactamase substrate CCF2/AM mix (K1024, Invitrogen) were added to the cells and incubated for 90 minutes at room temperature in dark. The results were analyzed with the SpectraMax iD5 Microplate Reader using the SoftMax Pro software.

13. Minicells adhesion assays to in vitro cultured Her14 cells and immunofluorescence microscopy.

Minicells with the desired SA specificity were harvested by centrifugation (4000 xg, 20 min) from minicells purifications stored at 4ºC in OptiPrep™ solution (Axis-Shield, Scotland) at 4% (w/v) in water, and resuspended at $4x10^8$ minicells/ml in DMEM. For a minicell-mammalian cell co-incubation at MOI 500:1, a 250 µl sample of this minicell suspension was added to a single well of a 24-well tissue culture plate containing the aforementioned Her14 cell line (~10^5 cells/well) that were grown on sterile coverslips (13 mm diameter, Thermo Fisher) placed at the bottom of the well. After four hours of co-incubation at 37 ^oC, the coverslips were washed by immersion 10 times in a large volume of PBS (100 ml) to remove unbound minicells. Subsequently, the coverslips were treated as in tumor cell lines immunofluorescences carried out in bacterial adhesion assays (see Materials and methods, section 7) and examined by confocal microscopy (Leica TCS SP5 multispectral confocal system).

14. Minicells purification.

A 10-ml LB pre-inoculum of the minicell producer strain was grown during 8 hours at 37ºC with agitation (160rpm), after that, pre-inoculum was transferred to a flask containing one liter of fresh LB that was grown overnight at 37ºC with agitation (160rpm). Next day, this one-liter culture of minCD- mutant bacteria was subjected to initial differential centrifugation at 550 xg for 40 minutes at 4ºC to decrease the bulk of the bacterial burden. The resulting bacterial cell/minicell supernatant was centrifuged at 4000 xg for 20 min at 4ºC to harvest the bacteria/minicell content, which was resuspended in Optiprep (Axis-Shield PLC, Scotland) solution at 4% (w/v) (see below) and subjected to two sequential density gradient centrifugation steps using OptiPrep, an isotonic biologically compatible medium which is a sterile 60% (w/v) solution of iodixanol in water. Gradients were performed with OptiPrep solutions ranging from 20%

to 6% (w/v). After both density gradient centrifugations minicells were found in a low-density band, whereas bacteria were found in the pellet. The enriched minicell fraction still contained some residual bacterial cells that were eliminated after incubating the suspension in LB liquid media with agitation for 30 min at 37ºC to reactivate any residual bacterial cell and then for 90 minutes in LB liquid media containing gentamycin (100 µg/ml) at 37ºC with agitation to kill any live bacterial cell. Lastly, the antibiotic treated minicell enriched fraction, was centrifuged at 4000 xg for 20 min at 4ºC and resuspended in 4% (w/v) OptiPrep solution and subjected to an additional density gradient centrifugation step to eliminate any dead bacterial cell. Purified minicells were stored in OptiPrep 4% solution at 4ºC up to two months.

15. Minicells quantification.

A method based on minicell protein content determination was developed to quantify minicells. A volume equivalent to $6x10^8$ CFU was collected from an overnight culture of EcM1luxSAega grown statically at 37ºC, centrifuged at 4000 xg for three minutes, resuspended in 200 µl of PBS, sonicated (LabSonic B, Braun) for 40 seconds to release protein content and serially diluted using two as dilution factor. Protein content in all dilutions was determined with bicinchoninic acid (BCA) protein assay kit (ThermoScientific) providing an equation that correlates CFU and protein content. A 100-µl fraction of the purified minicells solution was treated in the same way to obtain a BCA-based protein content estimation in minicells. The equation that correlates protein content and CFU enable to determine the number of minicells from the data of protein content estimated for them, including a correction factor of five given the size difference between bacteria (2 µm) and minicells (0,4 µm). A yield of approximately 10^{10} minicells was routinely obtained from one liter culture of minCD-mutant bacteria. Acuraccy of this quantification method was confirmed by determining the number of minicells in a standard purification with Nanosight's NS300 device (Nanosight Ltd.), an alternative method that allows visualization, counting and tracking of nanoparticles in liquid media.

RESULTS

13.1. Characterization of anti-EGFR nanobodies selected by E. coli display

Previous work in our lab generated an E. coli display library of VHHs after immunization of two llamas (Lama glama) with human tumor cell line A431, which overexpresses EGFR. From this library a total of five different clones specifically binding the extracellular domain of EGFR (eEGFR) were selected and named from Vegfr1 to Vegfr5 (V. Salema, 2014 PhD thesis). After purification of the encoded Nbs, we characterized their affinity and the binding properties by Surface Plasmon Resonance (SPR). eEGFR-Fc was covalently immobilized to SPR-sensor chip at ca.1000 response unit (RUs) and dilutions of the purified Nbs were injected at different concentrations (from 0.14 nM to 100 nM) to determine the dissociation constant at equilibrium (K_D) and the kinetic constants of association (k_{on}) and dissociation (k_{off}). The resulting sensorgrams were overlaid, aligned and analysed using 1:1 Langmuir binding model (Figure 9).

According to this analysis, clone VEGFR1 showed the highest affinity for eEGFR-Fc (K_D=0.47 nM), followed by clones VEGFR2 and VEGFR3 with K_Ds of ca. 2 nM in both cases. Clones VEGFR4 (K_D =15.9 nM) and VEGFR5 (K_D =24.2 nM) showed a lower affinity for eEGFR-Fc.

Next, we investigated whether these Nbs recognize different epitopes of EGFR by analizing their competition for binding to eEGFR-Fc. In this assay the Nbs were sequentially injected over the SPR sensor chip in order to record their binding in the presence of pre-bound Nbs. As a result we realized that VEGFR1 does not interfere with the binding of all other Nbs, indicating that is binding a different epitope of eEGFR not recognized by the other clones. Also Nb VEGFR5 binding was also independent of the other Nbs. VEGFR2 blocks the binding of VEGFR3 and VEGFR4 and vice-versa, meaning that they bind overlapping epitopes.

Therefore, these data suggest that at least three different epitopes are recognized by the selected anti-EGFR Nbs, one by VEGFR1, a second one by clones VEGFR2, VEGFR3 and VEGFR4, and a third one by VEGFR5. The competition assay is shown in the Figure 10.

1.1 Competition of EGF and nanobodies for EGFR binding.

We investigated whether the selected Nbs could compete with EGF ligand for binding to EGFR by SPR. Purified Nbs VEGFR1 to VEGFR5 (100 nM) were injected

onto an eEGFR-Fc sensor chip in the presence or absence of EGF (300 nM). The sensorgrams showed that EGF does not affect the binding of Nbs VEGFR1 and VEGFR5, whereas it reduces the binding of Nbs VEGFR2, VEGFR3 and VEGFR4 to roughly half (Figure 11).

Since clones VEGFR2, VEGFR3 and VEGFR4 compete with each other and with EGF these Nbs should likely bind to an epitope partially overlapping the EGF-binding site of EGFR or inhibit the conformational change necessary for the receptor to bind EGF (Schmitz et al., 2013). On the contrary, Nbs VEGFR1 and VEGFR5 bind to two distinct epitopes of EGFR that are independent of EGF binding.

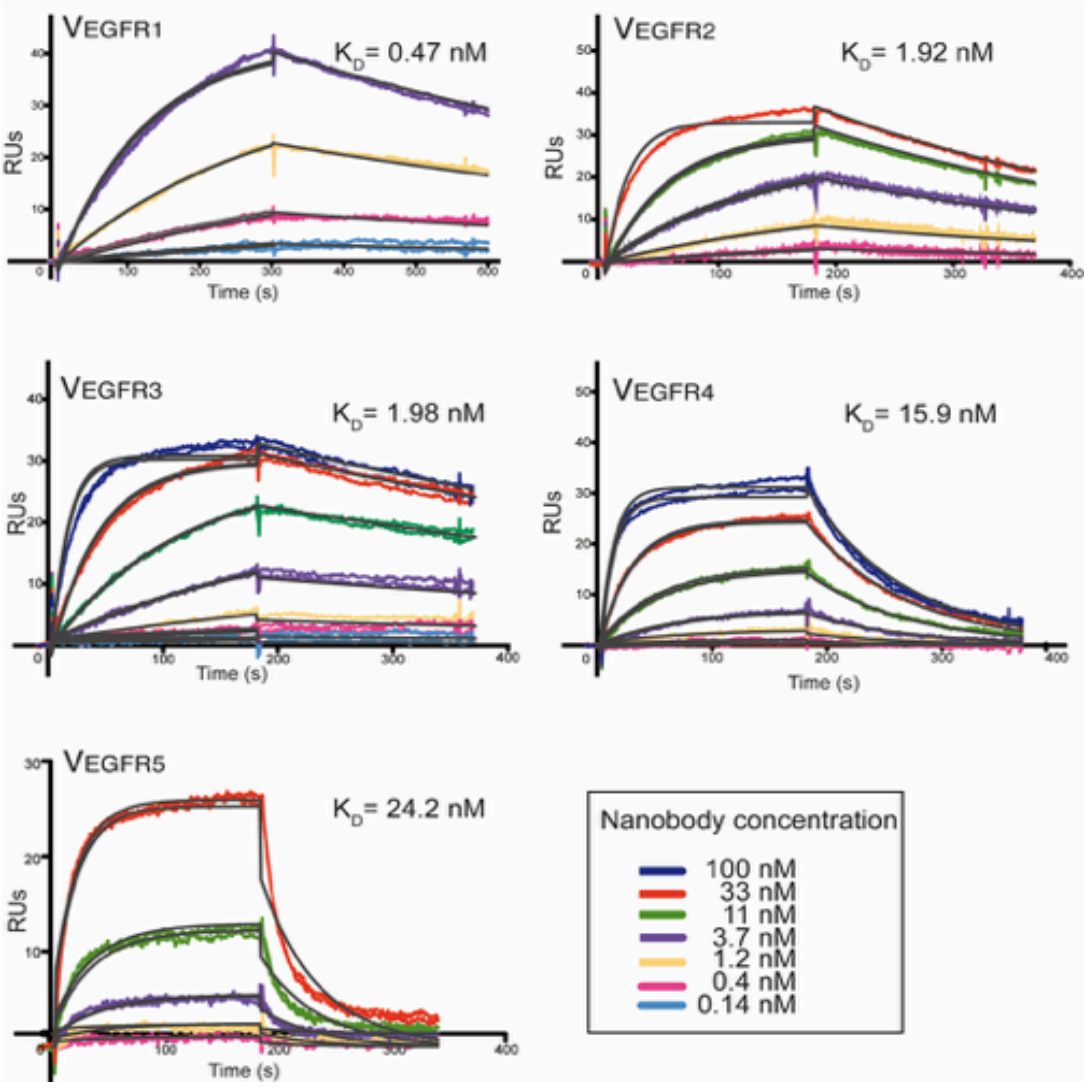


Figure 9. Determination of the equilibrium dissociation constant (K_D) of the selected anti-EGFR Nbs using surface plasmon resonance. SPR sensograms monitoring real-time association and dissociation of purified Nbs VEGFR1-5 (at concentrations indicated in the color code box) to eEGFR-Fc immobilized onto CM5 sensor chip. The increase in resonance units (RUs) was recorded along time (in seconds) and dissociation of Nbs was evaluated after the injection of the buffer. Sensograms from 2 independent injections are shown. The curve was fitted by non-

linear least squares regression using the 1:1 Langmuir binding model.

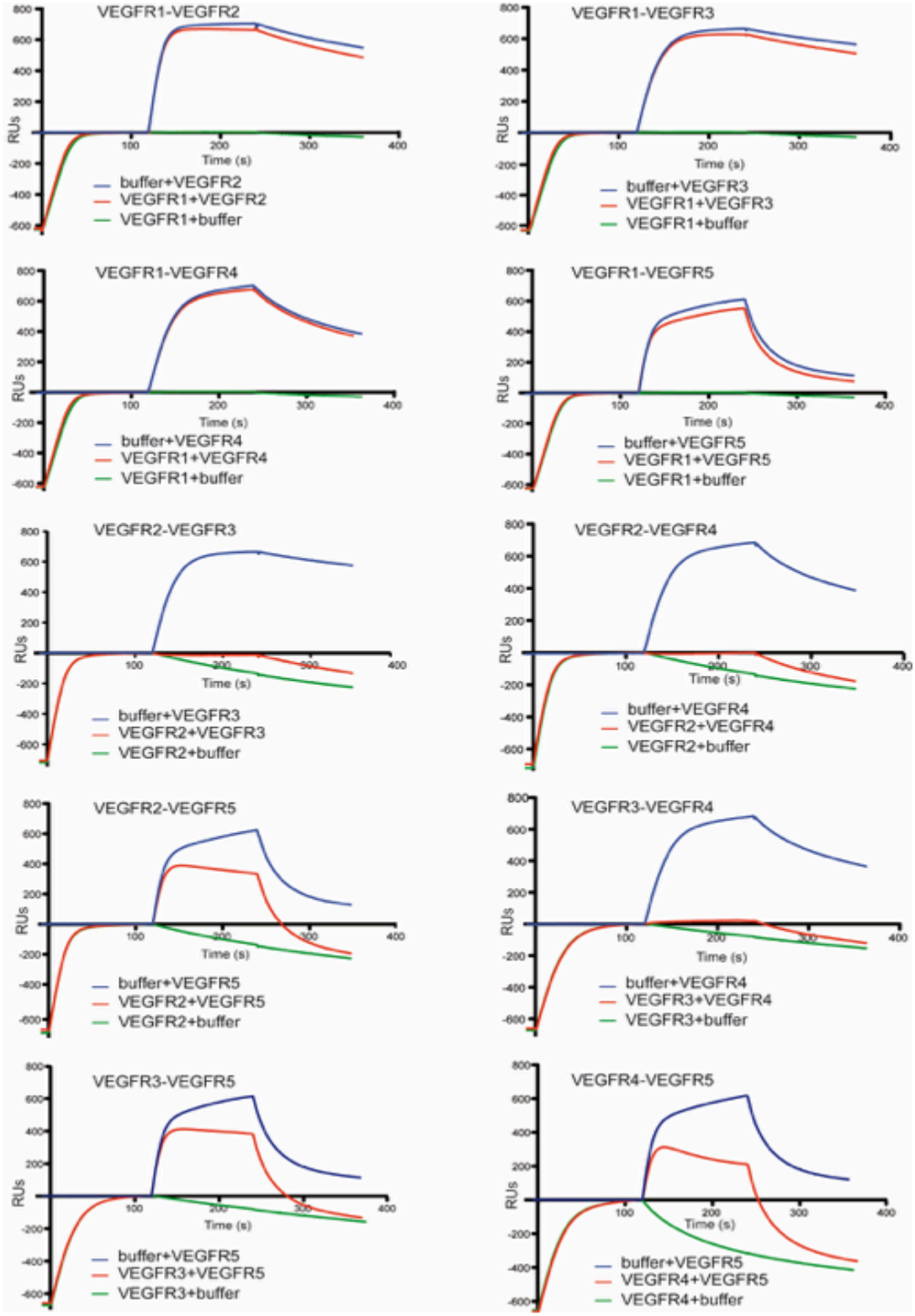


Figure 10. Competition of the selected Nbs for binding eEGFR by SPR. Epitope scouting analysis of the selected anti-EGFR Nbs (VEGFR1-5) carried out by SPR in pairwise manner to identify Nbs that recognize distinct or overlapping epitopes. The eEGFR-Fc antigen is immobilized on to the sensor chip and the first analyte (Nb1) is passed over the chip to allow

binding to the antigen observed by an increase in response units (RUs) in the sensorgram. Subsequently, the second analyte (Nb2) is applied to the Nb1-eEGFR-Fc complex to examine its binding. If the second analyte binds a separate epitope, additional binding and hence a subsequent increase in RUs in the sensogram is observed. All possible pairwise combinations of the Nbs were analyzed. Data show the more relevant combinations with the indicated Nbs.

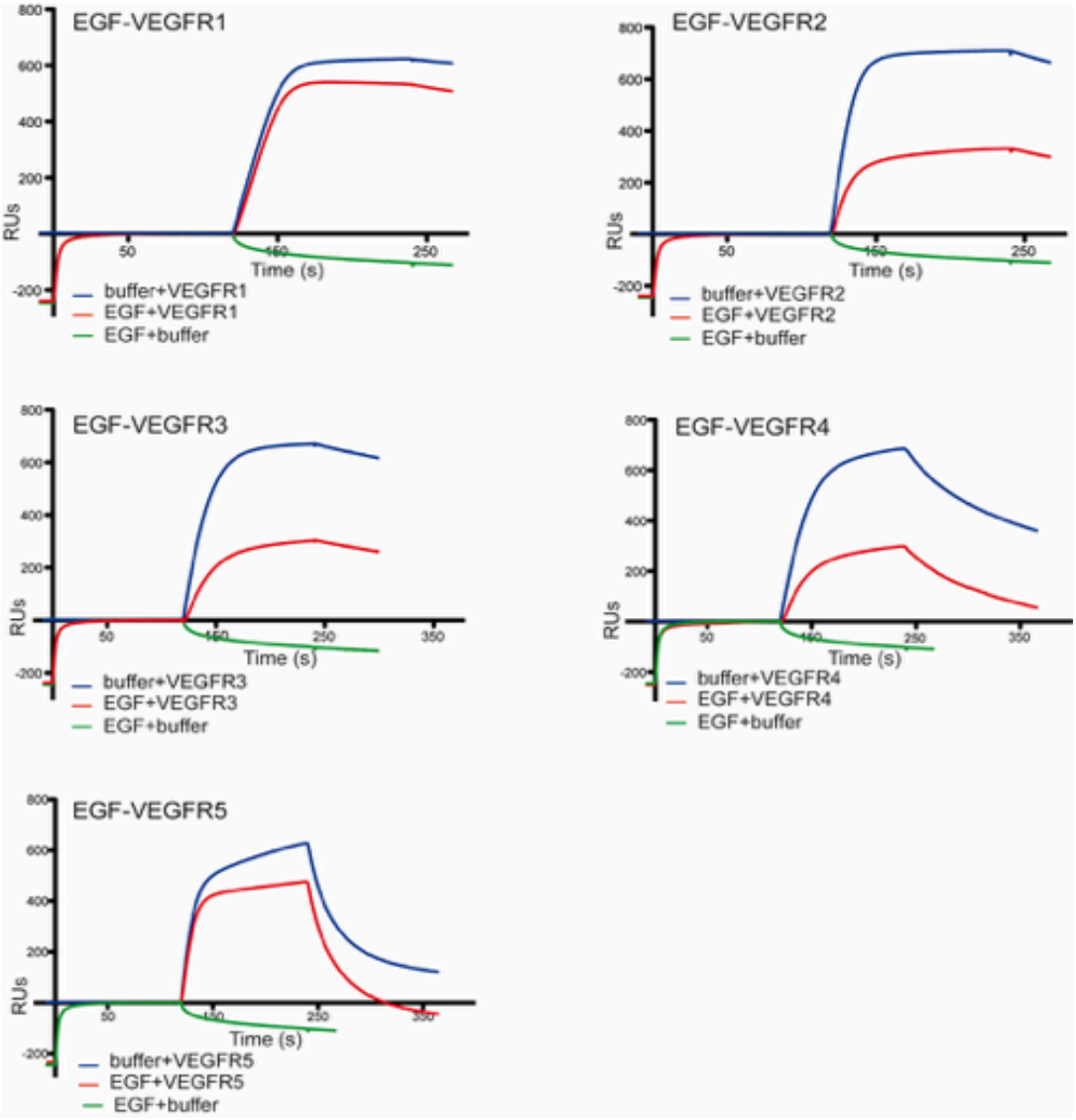


Figure 11. Competition of EGF and the selected Nbs for binding to eEGFR-Fc determined by SPR. SPR sensograms monitoring real-time association and dissociation of the indicated purified Nb (VEGFR 1-5) at 100 nM to eEGFR-Fc immobilized onto CM5 sensor chipin the presence or absence of pre-bound EGF (300nM). Sensograms are aligned at the point of Nb injection. Since EGF start to dissociate from eEGFR-Fc at the time of the Nb injection (see sensogram-buffer), this reduction in RUs of the sensrgrams with Nbs when EGF was pre-bound does not indicate competition. This is the case of Nbs VEGFR1 and VEGFR5. Higher reductions in RUs when EGF was pre-bound are indicative of EGF competition, as is the case for Nbs VEGFR2, VEGFR3 and VEGFR4, whose binding is partially abrogated in the presence of pre-bound EGF.

1.2 Effects of Nbs VEGFR1 and VEGFR2 on ligand-induced EGFR phosphorylation.

EGF and other ligands of EGFR can stimulate cell proliferation, cell differentiation, cell growth, migration, and inhibit apoptosis. When the Nbs bind to the extracellular domain of this receptor, exist the possibility that the binding alters the phosphorylation state of EGFR.

To characterize the effects of the Nbs in EGFR phosphorylation a WB with anti-Phospho-EGFR mAb was performed. Her14 cells were treated with EGF, VEGFR1 and VEGFR2 separately and with the combination between EGF and the different Nbs.

Incubation with EGF (15nM) results in the phosphorylation of the receptor, while the Nbs by themselves are not able to phosphorylate the EGFR (Figure 12).

This experiment also shows a partial blocking of the EGFR phosphorylation by the VEGFR2. This is likely due to a direct competition of EGF binding caused by VEGFR2, which may reduce the activation of the receptor.

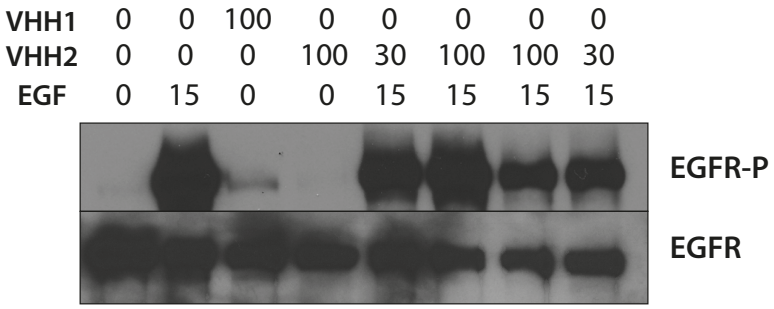


Figure 12. WB analysis of phosphorylated EGFR (upper lane) and EGFR (bottom lane) and upon treatment with Nbs VEGFR1 and VEGFR2. EGFR was detected with anti-EGFR mAb and anti-mouse IgG mAb-POD. Phosphorylated EGFR (EGFR-P) with a polyclonal anti-EGFR-P (Y1068) and anti-rabbit IgG-POD. The different concentrations of EGF and Nbs used are indicated (in µM).

1.3 Construction of synthetic adhesins against EGFR and expression on E. coli surface.

For tumor targeting the Nbs binding EGFR must be displayed on the bacterial surface allowing the bacteria to recognize the tumor cells expressing the receptor. Previous work in our lab describes the effective display of nanobodies in bacterial surface using the Intimin ß-domain that anchored the Nb to the bacterial OM (Salema et al., 2013a). The resulting fusion protein was named synthetic adhesin and is essential for targeting the tumor cells. The synthetic adhesin structure is shown in the Figure 4.

The existence of an E. coli strain already presenting a synthetic adhesin against GFP integrated the chromosome, replacing the flu gene (Pinero-Lambea et al., 2015a) allow us to generate new strains expressing SAs with other specificities by simply exchanging the Nb from the construction.

This was performed thanks to the pGERecomb-Vegfr1 and pGERecomb-Vegfr2 suicide plasmids, able to change just the Nb from the previous integrated SAgfp (Material and methods, Figure 6).

The E. coli strain used to express SAs is called EcM1lux. This strain has a deletion of the fim operon, encoding the type1 fimbriae, a major adhesin found in most E. coli strains. In addition, it carries the lux operon from Photorhabdus luminescens integrated replacing the mat operon encoding the conserved Mat fimbriae, also called E. coli common fimbriae (ECF) (Rendon et al., 2007).

Cultures of the resulting strains, EcM1luxSAegfr1 and EcM1luxSAegfr2, grown without any inducer or antibiotics, were analyzed by flow cytometry with anti-myc mAb to determine the expression levels of the SAs integrated in these strains. Flow cytometry data indicated that both SAs against EGFR were displayed on the bacterial surface with homogeneous levels in the population (Fig. 13).

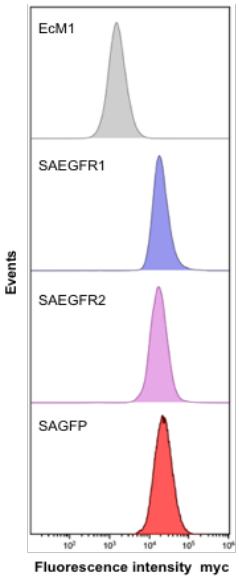


Figure 13. Events. Surface display levels of SAs in E. coli. Flow cytometry analysis of E. coli EcM1luxΔflu, EcM1luxSAegfr1, or EcM1luxSAegfr2 bacteria. Histograms show the fluorescence intensity of bacteria stained with anti-myc mAb and secondary anti-mouse IgG-Alexa 488.

2. Specific adhesion of engineered E. coli strains to cells lines expressing human EGFR.

2.1 Specific adhesion of engineered E. coli strains to transfected mouse fibroblasts expressing human EGFR.

The ability of EcM1luxSAegfr1 and EcM1luxSAegfr2 bacteria to adhere specifically to tumor cells expressing EGFR on their surface was evaluated. To this end, two isogenic cell lines derived from the mouse fibroblasts NIH 3T3 cells, termed 3T3#2.2 and Her14, were employed. 3T3#2.2 cells are devoid of endogenous mouse EGFR expression, while Her14 cells are stable transfectants of 3T3#2.2 cells expressing human EGFR on their surface (Honegger et al., 1987).

Subsequently, in vitro adhesion assays with EcM1luxSAegfr1 and EcM1luxSAegfr2 strains (MOI 300:1) to 3T3#2.2 and Her14 cells were performed. After one hour of infection unbound bacteria were removed washing with PBS, and the samples were fixed and stain with anti-*E. coli* polyclonal antibodies. Images of the adhesion assay showed that cells positive for EGFR surface expression (i.e. Her14) (red fluorescence) were largely covered by EcM1luxSAegfr bacteria (green fluorescence), while in cells negative for EGFR expression (i.e. 3T3#2.2) did not have bacteria attached. As a negative control another adhesion assay with EcM1luxSAgfp strain was performed under the same conditions resulting in a negative adhesion to Her 14 cells. Therefore, these data demonstrate that SAegfr1 and SAegfr2 enable specific attachment of bacteria to mammalian cells expressing human EGFR.

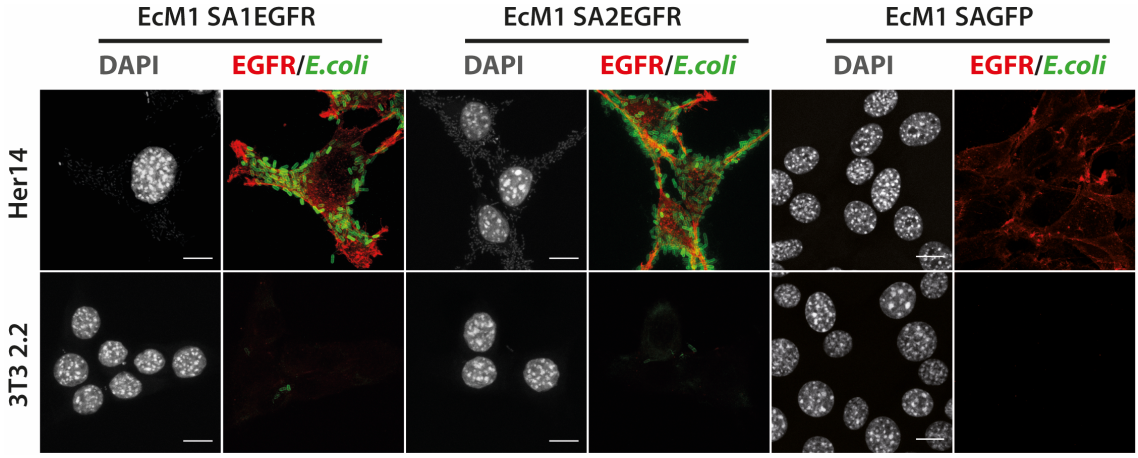


Figure 14. SAegfr1 and SAegfr2 drive the specific adhesion of E.coli to EGFR positive cell line. Confocal microscopy images of Her14 (top panel) and 3T3 2.2 cells (bottom panel) infected with EcM1luxSAegfr1 and EcM1lux SAegfr2 and EcM1luxSAgfp strains. Bacteria were stained with anti-E.coli polyclonal serum and anti-rabbit IgG-Alexa 488 (green), EGFR was stained with anti EGFR mAb and anti-mouse IgG-Alexa-594 (red), cell nuclei were stained with DAPI (grey).

Scale bars (white lines) represent 10 μm.

2.2 EGFR expression in bladder and colon cancer cell lines.

Next, we wanted to test whether bacteria with these SAs were able to adhere to human carcinoma cell lines expressing endogenous EGFR. Several bladder and colon cancer cell lines were employed. Flow cytometry analysis was used to estimate the expression of EGFR in bladder tumor cells belonging to different stages of cancer: stage I (RT112), stage III (T24 and UMUC 3), as well as colon cancer cell lines DLD1 and HCT116 both from primary human tumors. As controls we used Her14 cells (positive for EGFR) and 3T3 2.2 (negative for EGFR).

Flow cytometry data showed that all the cancer cell lines used in this assay were positive for expression of EGFR, although they expressed significantly less EGFR than Her14 cells (Figure. 15).

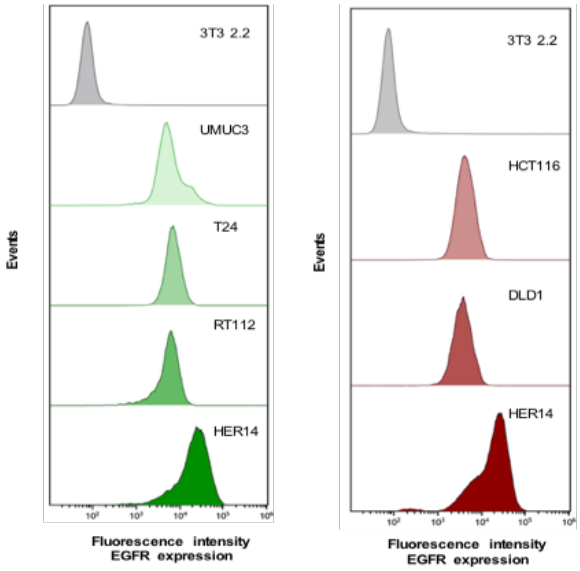


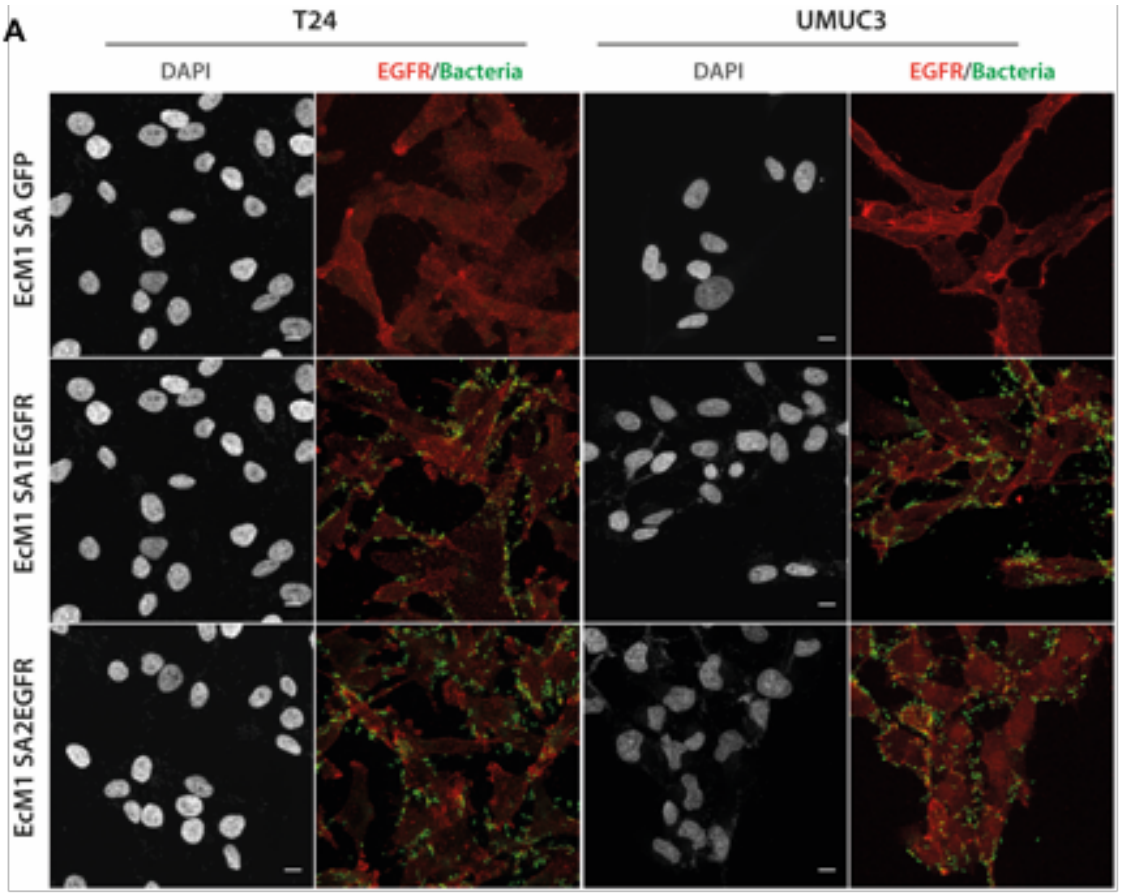
Figure 15. Flow cytometry analysis for EGFR expression on (A) bladder tumor cell lines RT112, T24, and UMUC3 and (B) on colon cancer cell lines HCT116 and DLD1. Mouse cell lines 3T3 2.2 and Her14 were used in A and B as negative and positive controls, respectively. Histograms show cell counts and fluorescence intensity with anti EGFR mAb and secondary anti-mouse IgG-Alexa 488.

2.3 Specific adhesion of the engineered *E. coli* strains to EGFR-positive bladder and colon tumor cells.

In order to determinate the capability of EcM1luxSAegfr1 and EcM1luxSAegfr2 to recognize the EGFR on bladder and colon tumor cells, we performed adhesion assays using the EcM1luxSAtir strain as a negative control.

T24 and UMUC3 bladder cell lines, and DLD1 and HCT116 colon cell lines, were employed to this experiment. Cells were infected for an hour with bacteria at a MOI of 300:1. Unbound bacteria were removed by washing with PBS, and samples were fixed

and stained with anti-*E. coli* polyclonal antibody. Inspection of the samples by confocal microscopy revealed high numbers of EcM1luxSAegfr1 or EcM1luxSAegfr2 bacteria (red fluorescence) specifically adhered to T24, UMUC3, DLD1 and HCT116 cells expressing the antigen EGFR (green fluorescence) on their surface. In the case of the colon cell lines, a higher number of bacteria were bound with SAegfr2 (Figure 16B). In contrast, EcM1luxSAgfp bacteria were not bound to those cells. Hence, both SAs enable specific adhesion of *E. coli* to EGFR-positive tumor cells (Figure 16). Since SAegfr2 appeared to produce higher adhesion in colon carcinoma cell lines, this SA was chosen for future experiments.


A
T24
UMUC3
DAPI
EGFR/Bacteria
DAPI
EGFR/Bacteria
EcM1 SA GFP
EcM1 SA1EGFR
EcM1 SA2EGFR

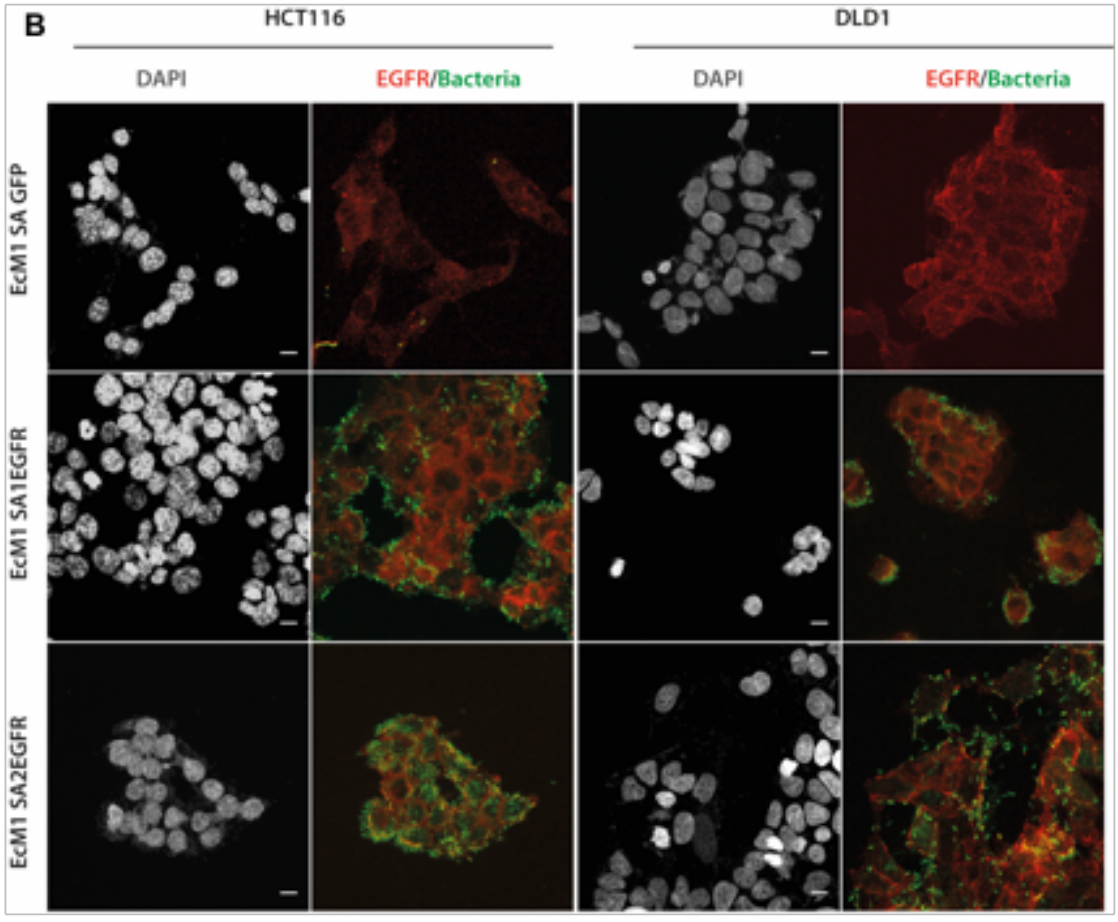

B
HCT116
DLD1
DAPI
EGFR/Bacteria
DAPI
EGFR/Bacteria
EcM1 SA GFP
EcM1 SA1EGFR
EcM1 SA2EGFR

Figure 16. Confocal fluorescence microscopy images of engineered *E. coli* strains targeting EGFR-positive tumor cells. A. Infection of bladder tumor cells T24 and UMUC3. B. Infection of colon tumor cells DLD1 and HCT116. Infection carried out with the indicated *E. coli* strains: EcM1luxSAegfr1, EcM1luxSAegfr2 and EcM1luxSAtir. Cell staining was carried out as in Figure 14. Scale bars (white lines) showed on the merge pictures represent 10µm.

14.3. Internalization and content release of bacteria into the tumor cells.

Once our bacteria were able to reach the tumor cells specifically, we investigated the use of bacteria as a vector for intracellular delivery of therapeutic proteins (e.g. cytotoxins) in the tumor cells. Previous studes showed that a combination of Invasin from Yersinia pseudotuberculosis and the listeriolysin (LLO) from Listeria monocytogenes can be expressed in *E. coli* to trigger bacterial internalization and the release of RNA or DNA molecules into the host cell cytoplasm (Critchley et al., 2004). We attempted to use this combination to deliver directly a protein cargo in tumor cells.

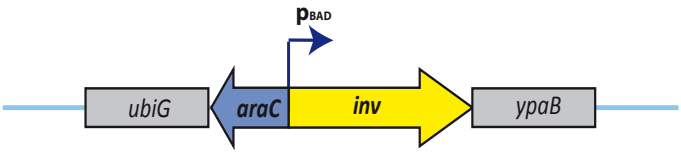
3.1 Invasin expression on E.coli surface.

Invasins are a class of proteins that permits the penetration of several pathogens into the host cells (Palumbo and Wang, 2006). In *Yersinia* species invasin is a fundamental virulence factor required for adherence and colonization of host tissues and is encoded in the inv locus in the bacterial chromosome (Dersch and Isberg, 2000; Gillenius and Urban, 2015).

Invasin recognizes ß-integrins expressed on several cell types like leukocytes or epitelial cells among others (Anderson et al., 2007). The protein structure consists in a ß-barrel-like domain anchored in the bacteria OM, a repeat of immunoglobulin-like domains and the C-terminal adhesion domain, which provides the invasin specificity to bind the target molecule ß-integrins.

Invasin used as a natural adhesin does not provide a specific targeting of our therapeutical bacteria against tumor cells due to the almost ubiquitous expression of ß-integrins in human cells (Desgrosellier and Cheresh, 2010). However, the controlled expression of invasin after the adhesion with SAs may allow us to invade the tumor cells that are specifically recognized by the synthetic adhesin. With this aim, inv gene was integrated replacing the yfaL gene of *E. coli* K-12, which encodes for an autotransporter (Wells et al., 2010). The suicide vector used, pGEyfaL::araC-P_{BAD}inv

(Figure 17), carries the inv gene under the control of the L-arabinose (L-ara) -inducibe



promoter P_{BAD}.

Figure 17. Gene construct for integration of invasin (inv) in the yfaL locus of E. coli K-12 under the control of araC-PBAD regulatory region.

To check the invasion of E. coli bacteria expressing inv under araC-P_{BAD}, we performed an internalization assay using the EcM1luxSAegfr2 strain carrying araC-P_{BAD}inv construct and expressing GFP in the cytoplasm for detection (*yeeJ-PtacGFP*). An immunofluorescence microscopy-based assay was used to differentiate between internalized bacteria and bacteria that remain attached to the tumor cell surface. In this assay, called "extracellular staining", cells infected with GFP-expressing bacteria are fixed but not permeabilized, so that only extracellular bacteria can be labeled with the anti-E. coli polyclonal serum (and secondary Ab conjugated with a fluorophore). Thus, intracellular bacteria are only labeled with GFP whereas extracellular bacteria are labeled with both anti-E. coli and GFP.

*EcM1lux*SAegfr2+inv+gfp strain was used for infection of HCT116 and T24 cells for 1 h. Non-adherent bacteria were removed by washing and L-ara was added to induce the expression of invasin. After 4 h, infected cells were washed, fixed and stained with anti-E.coli and secondary anti-rabbit IgG-Alexa 546, phalloidin-660 (actin) and DAPI (cell nuclei). Visualization of these samples by confocal microscopy (Figure 18) allowed to see internalized bacteria in green and extracellular baceria attached to the cell surface in yellow (green+red). In cells treated with L-ara, bacteria were found internalized in large clumps, likely due to the polymerization of the ß-integrins around bacteria in the cell surface. After 4 h induction of invasin, most bacteria are internalized into the tumor cells in samples incubated with L-ara. An important fraction of bacteria are also internalized in samples without L-ara, likely due to leaky expression of inv from P_{BAD} (Figure 17). However the internalization level observed is lower than that observed in samples with L-ara, and being characterized by the absence of clumps of internalized bacteria. Therefore, we conclude that there is a successful internalization of bacteria in tumor cells due to invasin expression. We also confirmed that invasin expression causes the polymerization of actin filaments surrounding internalized

bacteria (Figure 19). Observation of HCT116 cells 24 h post-infection, allowed to detect internalized bacteria with GFP fluorescence at this long incubation time, which suggest that bacteria are not lysed by epithelial cells (non-phagocytic) upon internalization (Figure 20).

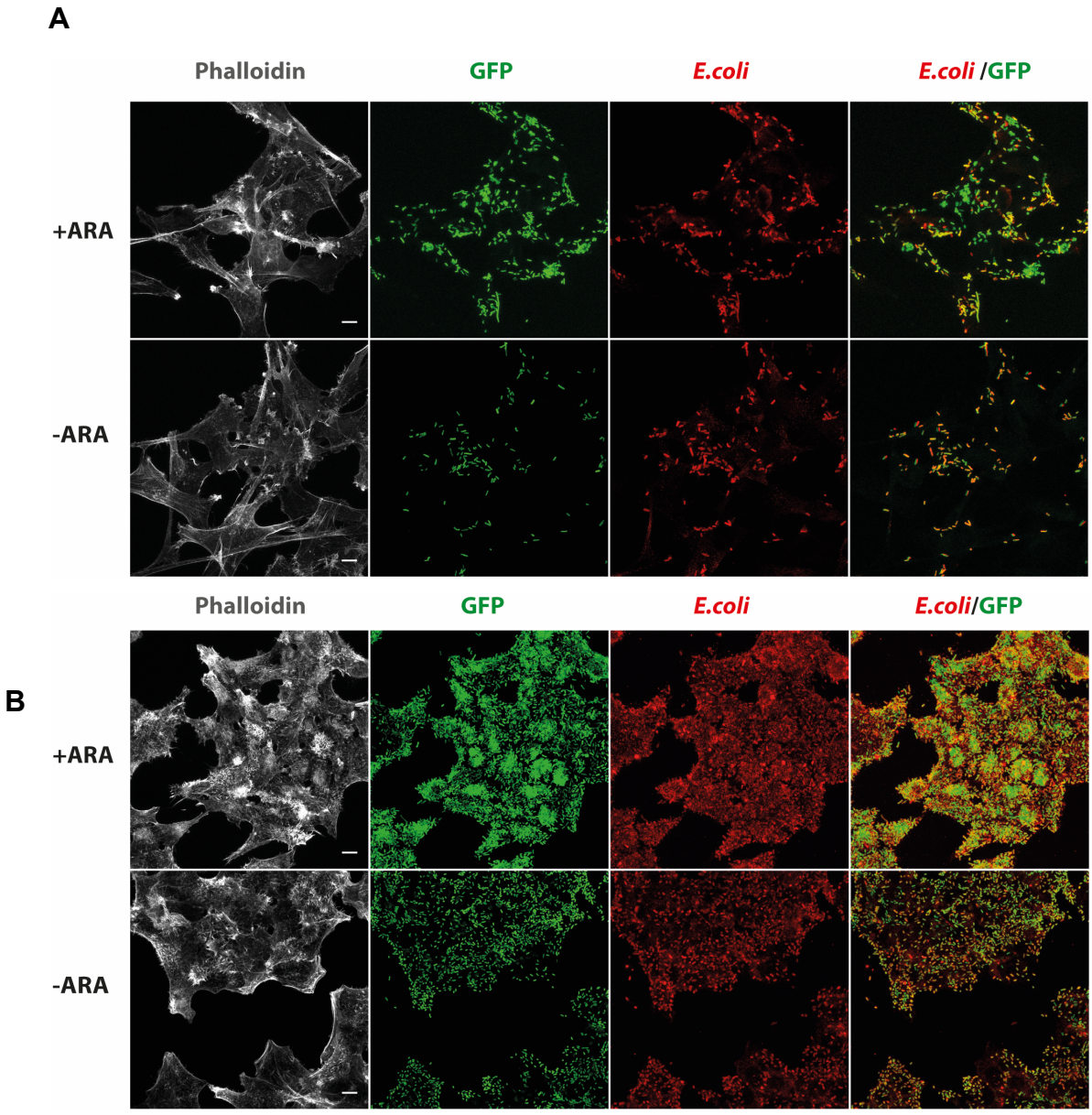


Figure18. Confocal microscopy of the adhesion and invasion of A. T24 bladder tumor cells and B. HCT116 colon tumor cells infected with EcM1luxSAegfr2+inv+gfp (MOI 100:1). Extracellular bacteria are stained with anti-E.coli polyclonal and secondary anti-rabbit Alexa 594 (red). All bacteria express GFP (green). F-actin in tumor cells is stained with phalloidin 660 (grey). The scale bars correspond to 10 µm.

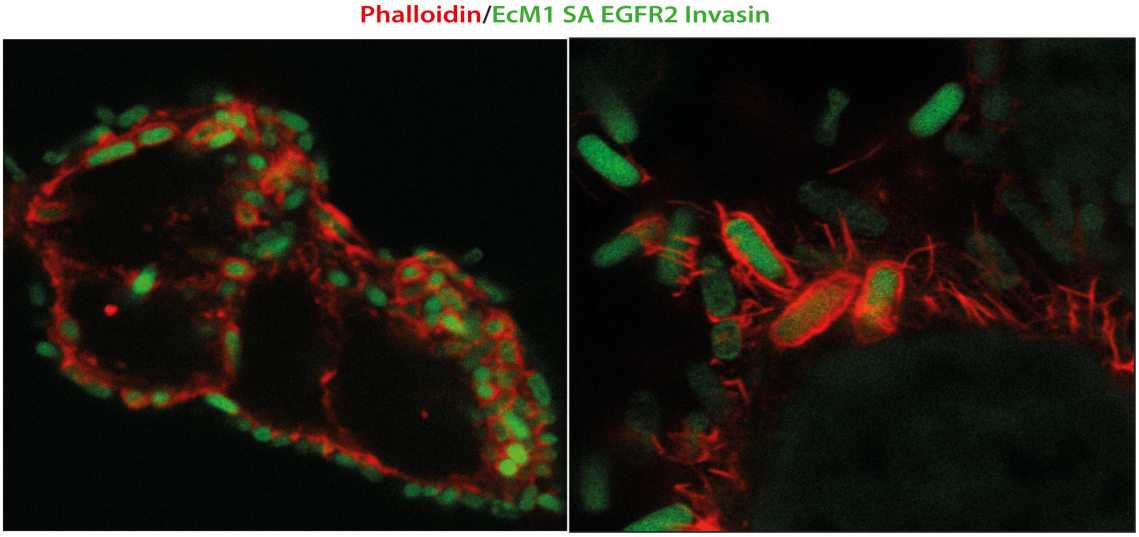


Figure 19. Invasin expression triggers actin polymerization around internalized bacteria. Confocal microscopy of actin polymerization during the adhesion and invasion of HCT116 cells by EcM1luxSAegfr2+inv+gfp (MOI 30:1). Samples stained as in Figure 18.

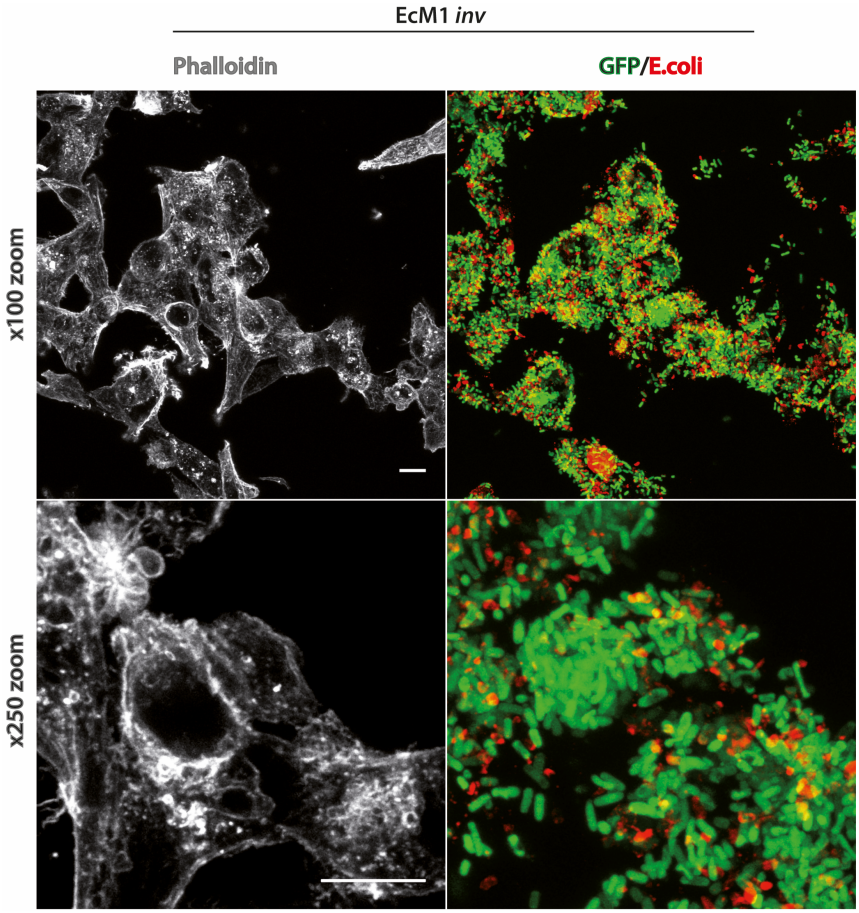


Figure 20. Confocal microscopy of the invasion of HCT116 colon tumor cells infected with EcM1luxSAegfr2+inv+gfp (MOI 100:1) for 24 h. Samples are stained as in Figure 18. The scale bar corresponds to 10 μm

3.2 Evaluation of apoptosis and cell death triggered by bacterial internalization

Next, we evaluated whether bacterial internalization was inducing tumor cell death and/or apoptosis using two different flow cytometry assays.

The LIVE/DEAD® Viability/Cytotoxicity Assay provides a two-color fluorescence cell viability assay that is based on the simultaneous determination of live and dead cells with two probes that measure intracellular esterase activity and plasma membrane integrity. Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually non-fluorescent cell-permeant calcein-AM to the intensely fluorescent calcein. The polyanionic dye calcein is well retained within live cells, producing an intense uniform green fluorescence. Ethidium homodimer-1 (EthD-1) enters cells with a damaged plasma membrane and stain nucleic acids producing a bright red fluorescence. EthD-1 is excluded by the intact plasma membrane of live cells. Figure 13 shows a flow cytometry analysis of HCT116 cells infected for 24 h with invasive bacteria EcM1luxSAegfr2+inv+gfp in the presence or absence of L-ara. In this assay, cultures are treated with gentamycin after 4h infection to kill extracellular bacteria and prevent their overgrowth in the culture.

Data obtained revealed that bacterial invasion of HCT116 cells produces only a moderate increase of cell death, from ca. 5.74 % in non-infected cells to ca. 15% in infected samples without L-ara and ca. 25% when L-ara is added (Figure 21).

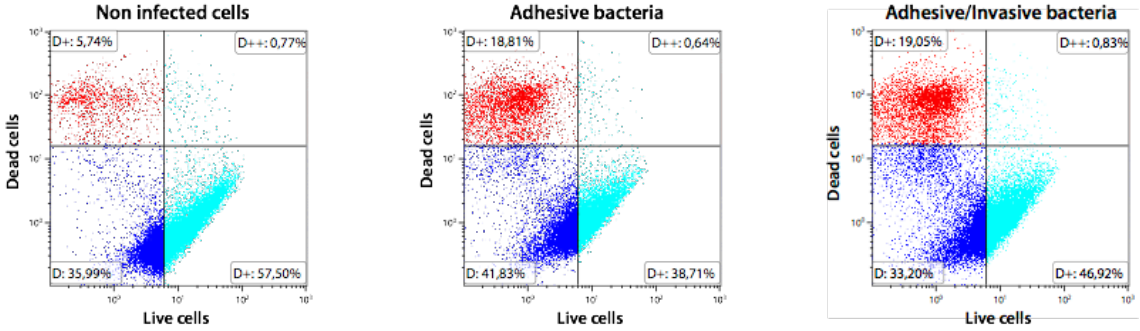


Figure 21. Cell death after bacterial invasion of HCT116 tumor cells. Flow cytometry of the LIVE/DEAD® Viability/Cytotoxicity Assay of HCT116 cells after 24h of infection with EcM1luxSAegfr2+inv+gfp in the presence and absence of L-ara, as indicated. Uninfected HCT116 cells are used as a control.

During apoptosis, phosphatidylserine (PS) is translocated from the cytoplasmic face of the plasma membrane to the cell surface. Annexin V has a strong, Ca^{2+}-dependent affinity for PS and therefore can be used as a probe for detecting apoptosis. We

performed an invasion assay of HCT116 cells with EcM1luxSAegfr2+inv+gfp, in the presence and absence of L-ara, adding gentamycin after 4 h of infection, and incubating samples for a total of 24 h. Cell samples from 4 h (before gentamycin) and 24 h infection were stained with Anexin V-APC and analysed by flow cytometry (Figure 22). This experiment shows that at 4 h p.i. those cell samples infected with bacteria had a strong GFP fluorescence signal, indicating an effective adhesion of bacteria to the HCT116 cells, and no induction of apoptosis, with levels of Anexin V-staining similar to the uninfected control (Fig. 22). In contrast, at 24 p.i. only the infected sample treated with L-ara showed a strong GFP fluorescence signal, as expected for the induction of invasin and the internalization of GFP-expressing bacteria, which are not killed with gentamycin (Fig. 22). In addition, the highest level of cell apoptosis (18,23 % Anexin V-positive cells) was found in samples infected with invasive bacteria, compared to uninfected control (2,51 % Anexin V-positive cells) However, the increase in Anexin-V-positive cells was no so significant in comparation with infected cells not incubated with L-ara (11,98% Anexin-positive cells). Hence, this experiment revealed that bacterial invasion of HCT116 tumor cells is very effective after induction of invasin, but this massive internaliation is still insufficient by itself to induce a major induction of cell apoptosis after 24 h.

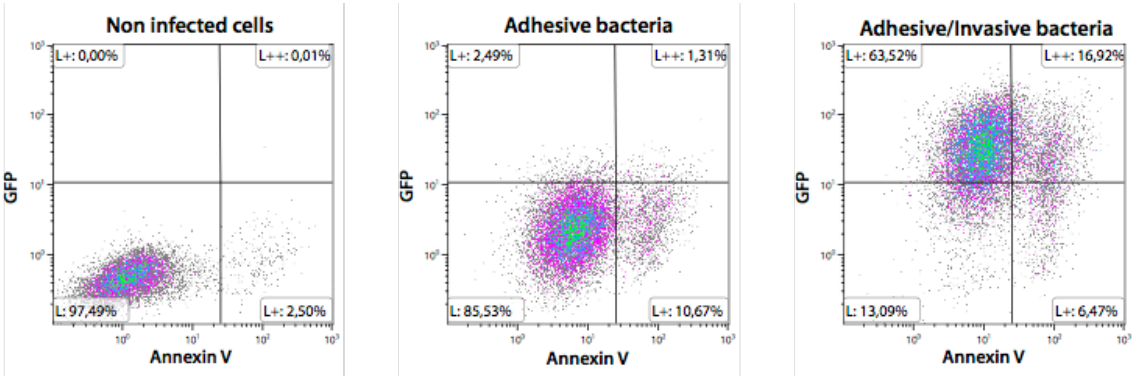


Figure 22. Apoptosis of HCT116 tumor cells after bacterial invasion. Flow cytometry assay of HCT116 cells at 4 h (A) and at 24h (B) post infection with EcM1luxSAegfr2+inv+gfp in the presence and absence of L-ara, as indicated. Samples stained with Anexin V-APC and GFPI. Uninfected HCT116 cells are used as a control.

3.3 Evaluation of Listeriolysin O (LLO) for the release of a protein cargo into host cells after bacterial invasion.

Intracellular pathogens have different mechanism to escape from the phagosome (Lerena et al., 2010) (Lam et al., 2011). E. coli K-12 does not have any of these mechanisms and, indeed, we observed our engineered invasive bacteria intact inside

the tumor cells after 24 h (Figure 20). To attempt the lysis of the phagosome for the release of a protein cargo expressed in bacteria, we engineered the expression of Listeriolysin-O (LLO) in the chromosome of our invasive E. coli strain.

LLO is a cholesterol-dependent cytolysin produced by the intracellular pathogen Listeria monocytogenes to escape from the phagosome into the host cell. A unique LLO feature is a low optimal pH<6 for escape form the phagosome without damaging the plasma membrane (Dramsi and Cossart, 2002). The llo gene was amplified from L. monocytogenes and cloned without its endogenous signal peptide to allow the accumulation of LLO in the cytoplasm of E. coli. In addition, we generated a periplasmic version of LLO with the pelB signal peptide form Klebsiella oxytoca (Francetic and Pugsley, 2005). Both gene constructs were inserted into chromosome of EcM1luxSAegfr2+inv+gfp substituting the fimbrial operon yraHIJK (Jiang et al., 2010) by using the suicide plasmids pGEΔyraP_{BAD}llo and pGEΔyraP_{BAD}pelBllo, respectively (Figure 23). Thus, addition of L-ara in the resulting strains (*EcM1luxSAegfr2Δinv*Δ*lloΔgfp* and *EcM1luxSAegfr2Δinv+pelB-lloΔgfp*) should result is the simultaneous expression of invasin and LLO in the bacteria.

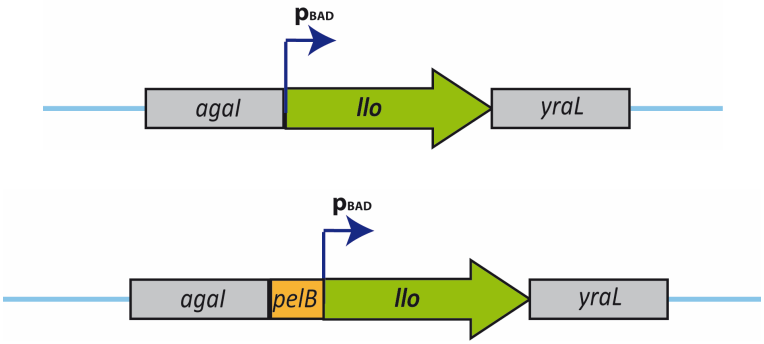


Figure 23. LLO constructs for integration in the yra site of E. coli chromosome under control of the P_{BAD} promoterwith and without the signal peptide pelB.

Lastly, since cytoplasmic LLO does not have access to the phagosome membrane before bacterial lysis, we deleted the dapA gene in *EcM1luxSAegfr2Δinv*Δ*lloΔgfp* to accellerate bacterial lysis. The dapA gene encodes the 4-hydroxy-tetrahydrodipicolinate synthase required for the syntheis of the essential peptidoglycan component diaminopimilic acid (DAP). Thus, ΔdapA strains are auxotrophic and present inestability of the cell wall in the absence of DAP in the medium (Critchley-Thorne et al., 2006).

To detect and quantify the release of a protein cargo in tumor cells after bacterial invasion, we used the reporter enzyme TEM-ß-lactamase (BlaM) devoid of signal

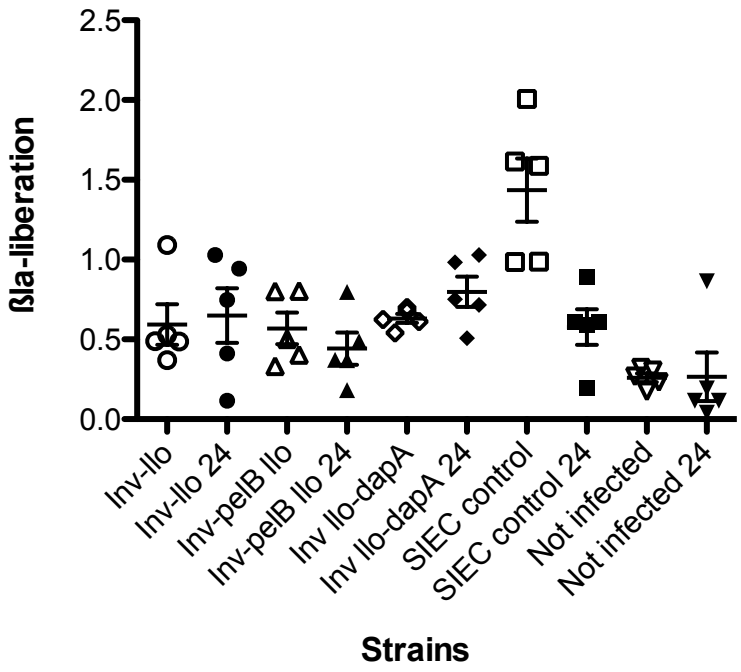
peptide. The activity of BlaM can be specifically detected and quantified in the cytoplasm of the mammalian cells, but not in the cytoplasm of the producing bacteria, with the substrate CCF2 (Charpentier and Oswald, 2004). CCF2 is a fluorescence resonance energy transfer (FRET) substrate, which consist of a cephalosporin core linking 7-hydroxycoumarin to fluorescein. CCF2-AM is a non-fluorescent lipophilic esterified form of CCF that allow its readily entry into cells. Cytosolic esterases specifically present in mammalian cells rapidly converts CCF2-AM into its negatively charged form, CCF2, which is retained in the cytosol.

In the absence of BlaM activity, exciting the coumarin at 409 nm in the intact CCF2 molecule results in FRET to the fluorescein, which emits a green fluorescence signal at 518 nm. In the presence of BlaM activity, however, cleavage of CCF2 disrupts FRET, so that exciting the coumarin at 409 nm now produces a blue fluorescence signal at 447 nm. This blue signal can be detected as an increase in the blue channel readout on fluorescence microplate readers.

For this experiment we seeded HCT116 cells in 96 wells plates at 80% of confluency, and were infected with our bacterial strains expressing inv and llo (Inv-llo), inv and pelB-llo (Inv-pelBllo), the auxotrophic mutant Inv-llo-ΔdapA. These strains were transformed with the pBAD18-BlaM that expresses cytoplasmic BlaM under control of the L-ara inducible araC-P_{BAD} promoter. Infection with these strains (MOI 30:1) in the presence of L-ara was conducted for 4 h and 24 h, in the latter case adding Gentamycin after 4 h. As a positive control we used the Synthetic Injector *E. coli* (SIEC) strain previously constructed in our lab (Ruano-Gallego et al., 2015), which encodes the injectisomes of the type III secretion system (T3SS) of enteropathogenic *E. coli* (EPEC). The SIEC strain was transformed with the plasmid pBAD18-T3S-BlaM that encodes a fusion of a T3S-signal to BlaM for the direct injection of the enzyme into the cytosol of mammalian cells through the injectisomes (Charpentier and Oswald, 2004). Infection of HCT116 cells with SIEC (MOI 30:1) was conducted for 4 h adding IPTG and L-ara to induce the expression of T3SS injectisomes and T3S-BlaM, respectively. In addition, a 24 h sample of this infection was also obtained by addition of Gentamycin after the initial 4 h of infection. Treatment with Gentamycin kills extracellular bacteria, as is the case of SIEC bacteria (Ruano-Gallego et al., 2015). All samples, from 4 h and 24 h infections, were incubated with CCF2-AM for 90 min and their fluorescence emission at 447 nm and 518 nm were measured. The results from these assays shows that, after 4 h infection, only the cells infected with the positive control SIEC strain, and none of invasive strains, had a clearly detectable BlaM activity in the cytosol above the background of uninfected cells (Figure 24). After 24 h

infection, however, cells infected with the invasive strains, especially Inv-llo-ΔdapA strain, showed a weak increase in the BlaM activity above the background levels of uninfected cells (Figure 24). At 24 h infection time, only a residual activity of BlaM was detected in cells infected with SIEC. This was expected due to the Gentamycin treatment, which killed SIEC bacteria at 4 h of infection and then the injected BlaM levels are likely diluted due to proteolysis and cell growth. Hence, the invasive strains with LLO, especially the Inv-llo-ΔdapA strain, are able to release a protein cargo in the cytosol of tumor cells after 24 h of infection, although at low levels, representing ca. 15 to 30% of those injected in 4 h by the SIEC strain.

Figure 24. Graphic for translocation of ßlactamase to the cytoplasm of HCT116 cells infected for 4 hours or 24 hours with the tested invasive bacterial strains, As a control we measured the injection of ßla by the SIEC strain and the non–infected cells.



3.4 Induction of a protein cargo in bacteria after invasion of tumor cells.

We also checked the capability of the internalized bacteria to induce a protein cargo. As a proof-of-concept the gfp gene was cloned under control of the tetR-PtetA promoter (Bertram and Hillen, 2008) for induction with anhydro-tetracycline (aTc). The construct was cloned in the suicide vector pGEypjA::tetR-PtetA-gfp for insertion in the chromosome of *E. coli*, replacing ypjA which encodes an autotransporter (Roux et al., 2005).

An adhesion-invasion experiment of HCT116 cells was performed with L-ara for 4 h followed by Gentamycin treatment for additional 1 h at 100µg/ml. Next, aTc was added or not (as a control) and infection continued for additional 2 h. Samples were fixed and stained with anti-*E. coli*, phalloidin, and DAPI (Figure 25). This experiment showed that intracellular bacteria strongly expressed GFP, demonstrating that a protein cargo can be induced in the bacterium after cell invasion.

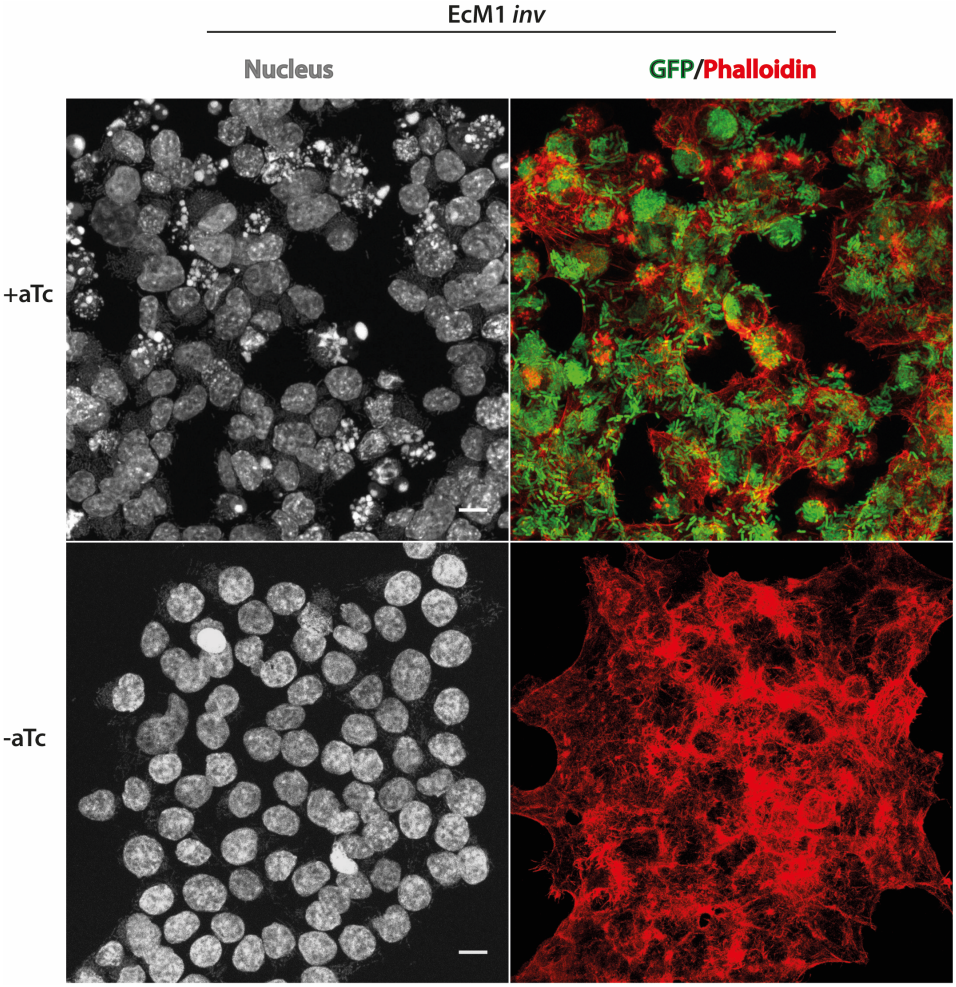


Figure 25. Induction of a bacterial protein cargo in the phagosome of tumor cells. Confocal microscopy of HCT116 colon tumor cells infected with EcM1luxSAEGFR2PBAD-invPtet-gfpΔypjA::TetRPtet gfp (MOI 100:1) for 4 h in the presece of L-ara and with gentamycin for additional 1 h. Then, aTc was added or not (control) to induce GFP expression in the internalized bacteria. Samples stained with phalloidin and DAPI to visualize actin (red) and cell nuclei (grey). Bar = 10 µm.

4. Synthetic adhesins against EGFR-tumor cells in non-live E. coli minicells.

In addition of live bacteria as delivery vectors for therapeutic molecules to tumor cells, non-live bacterial derived minicells could be appropriated for this aim (Flemming, 2007; MacDiarmid et al., 2007; Tsuji et al., 2016; Xin et al., 2017). An E. coli strain producing minicells (*EcM1*Δ*minCD* Δ*fliCD* Δ*msbB*), called *EcMini*, was previously constructed in the laboratory (Piñero-Lambea, 2014, PhD thesis). Deletion of minCD results in the generation of minicells. Deletion of *fliCD*, encoding the flagellin and flagellar cap protein, and msbB (also known as *lpxM*), responsible of the normal hexa-acylation of lipid A, reduce the innate immune response against flagella and LPS (Brubaker et al., 2015; Rosadini and Kagan, 2017; Somerville et al., 1999). The EcMini strain was used to generate minicells expressing SAs against EGFR on their surface and a protein cargo in their cytoplasm.

4.1 Generation of minicells with synthetic adhesins and a cargo protein.

SAs binding human EGFR and Tir from EHEC, as negative control, were integrated at the flu site of *EcMini* chromosome (Material and Methods 2).

Minicells were isolated in the supernantat from cultures of *EcMini-SAegfr* and *EcMini-SAtir* strains by differential centrifugation at low speed followed by density gradient centrifugations. Lastly, a Gentamycin treatment was used to kill any residual bacteria (Material and Methods 14). Purified minicells preparations were plated in LB agar to confirm that they were free of viable bacteria (data not shown). Using this methodology, we routinely purified ca. 6 x 10^9 minicells per liter of culture at OD600=1.

Western blot analysis of purified minicells and producing bacteria (Figure 26) confirmed the presence of SAs anti-EGFR and anti-Tir in the minicells of EcMiniSAegfr and EcMiniSAtir, respectively, and not in the parental strain EcMini. We also confirmed that minicells contained normal levels of bacterial proteins such a OmpA (OM porin) and GroEL (cytoplasmic chaperonin). Lastly, we demonstrated the display of SAs anti-EGFR and anti-Tir on the surface of the purified minicells EcMiniSAegfr and EcMiniSAtir by incubation with anti-myc mAb and secondary anti-mouse IgG-Alexa 488 (green) followed by fluorescence microscopy (Figure 27). Therefore, the purified minicells are made up of the protein content of the parental bacterial cells and display the SAs on their surface.

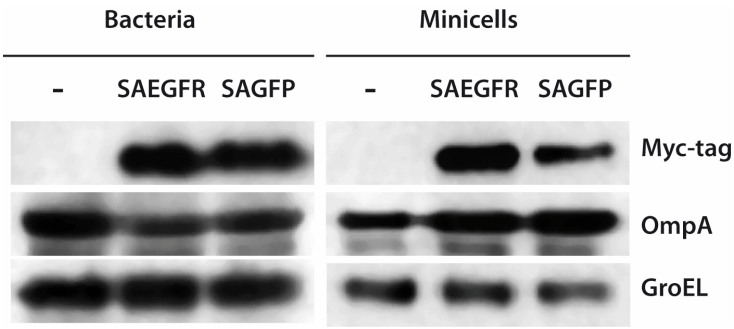


Figure 26. Synthetic adhesins expressed in E. coli minicells A. Western blot of complete protein extracts from bacteria and purified minicells (from the indicated strains) probed to detect: SAs (anti-myc mouse mAb and anti-mouse IgGs-POD; top panel), OmpA porin (anti-OmpA rabbit polyclonal serum and anti-rabbit IgG-POD; middle panel), and GroEL chaperonin (anti-GroEL mAb-POD). B. Fluorescence microscopy images of purified minicells from EcMini, EcMiniSAegfr, EcMiniSAtir strains, as indicated, stained with anti-myc mAb and secondary anti-mouse IgG-Alexa 488 (green) to detect SAs on the surface of the minicells

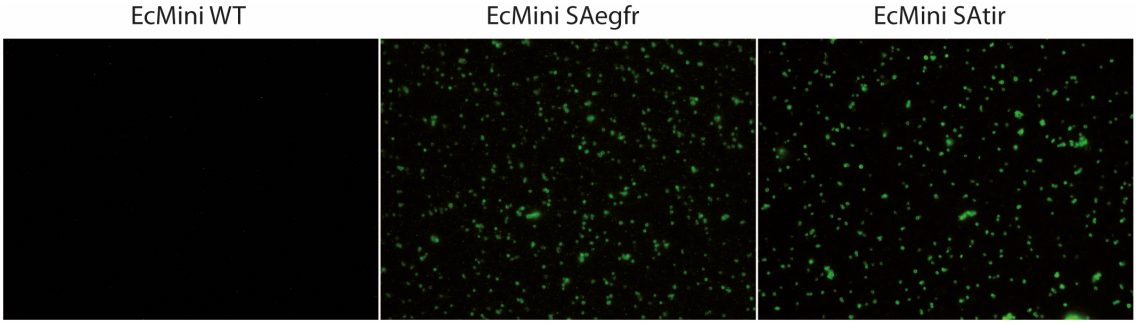


Figure 27. Minicells with GFP as a cargo protein. Bright field (left) and fluorescence microscopy (right) images of minicells purified from EcMiniSAegfr-Ptac-gfp strain induced with IPTG showing GFP expression in the cytosol.

Next, we generated "cargo-loaded" minicells using GFP as a model cargo protein. A Ptac-gfp construct was inserted in the chromosome of EcMiniSAegfr by substitution of yeeJ locus using the suicide vector *pGEΔyeeJ::Ptacgfp*. During the bacterial growth we are able to accumulate the GFP in the bacterial cytoplasm by induction with IPTG and the minicells obtained after purification (*MiniSAegfr-GFP*) contained GFP in the cytoplasm (Figure 28).

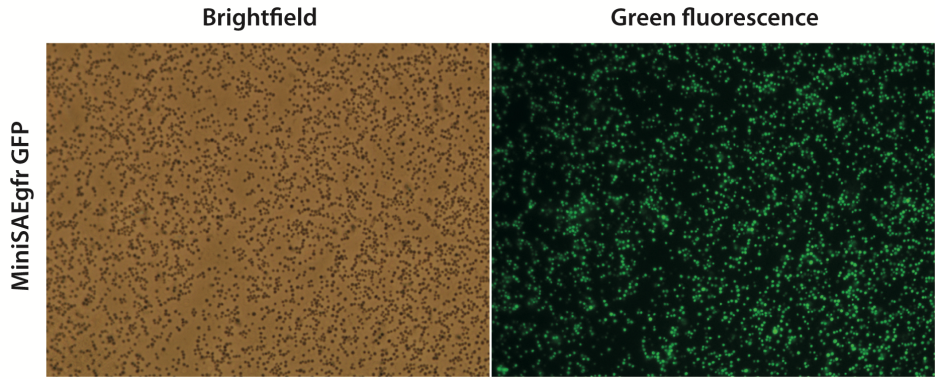


Figure 28. Adhesion of minicells with synthetic adhesins to Her14 (EGFR+) and 3T3 2.2 (EGFR-) cells. Confocal images of Her14 and 3T3 2.2 cells incubated with minicells MiniSAegfr-GFP and MiniSAtir-GFP for 4 h (MOI 500:1). Samples were stained with anti-EGFR (red) and DAPI (grey). Minicells contain GFP (green).

15.1.1 4.2. Adhesion of the engineered minicells to tumor cells

We tested the capability of the engineered minicells to recognize and bind the target molecule on the surface of tumor cells. Minicells from E. coli strains EcMiniSAegfr-Ptac-gfp and EcMiniSAtir-Ptac-gfp, as a negative control, were incubated for 4 h with Her14 (EGFR+) and 3T3 2.2 (EGFR-) cells (MOI 500:1). Unbound minicells were washed and the samples were fixed, stained with anti-EGFR mAb and DAPI, and inspected by fluorescence microscopy (Figure 28). This experiment revealed that only a few minicells MiniSAegfr-GFP adhere to Her14 cells. In addition, a similar number of minicells were bound to the control cell line (3T3 2.2) and minicells (MiniSAtir-GFP).

To test whether longer incubation times could improve adhesion, we performed an identical in vitro adhesion assay to Her14 cells incubated with MiniSAegfr-GFP and MiniSAtir-GFP for 4 h and 16 h (Figure 30). Although a higher number of minicells MiniSAegfr-GFP were able to bind Her14 cells at 16 h, adhesion of control minicells MiniSAtir-GFP also increased. Hence, despite the higher MOI and longer times of incubation, these experiments indicated an apparent inability of minicells with SAs to bind effectively to target cells. This result dramatically contrast with our previous data using live *E. coli* bacteria expressing SAs (Figure 14).

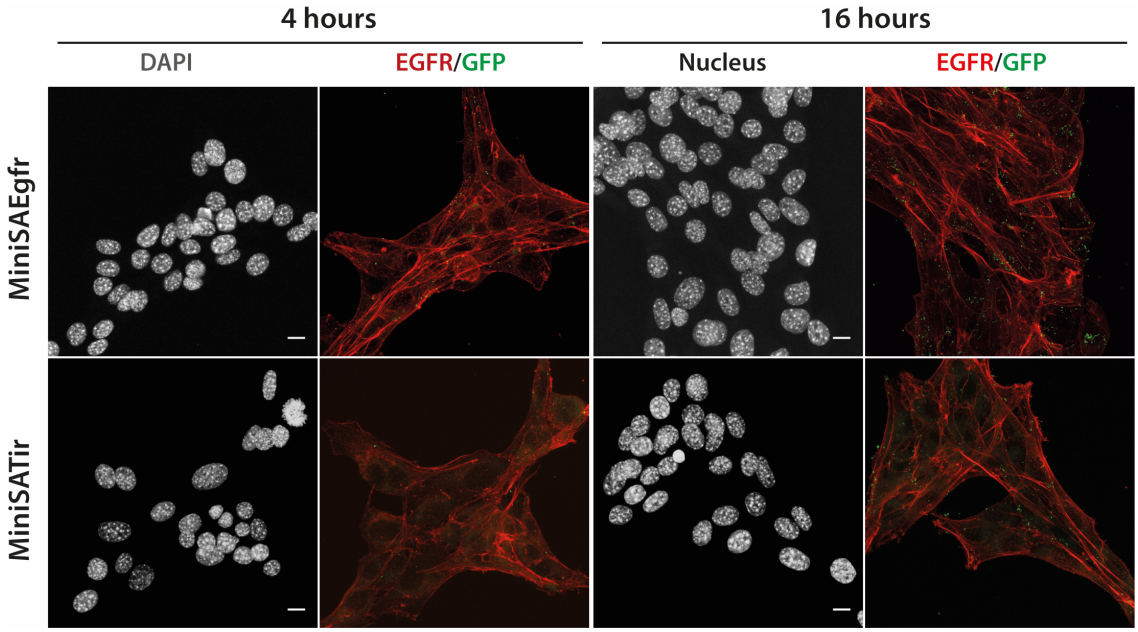


Figure 29. Adhesion of minicells with synthetic adhesins to Her14 (EGFR+) cells at long incubation times. Confocal images of Her14 cells incubated with minicells (MOI 500:1) MiniSAegfr-GFP and MiniSAtir-GFP for 4 h (left) and 16 h (right). Samples were stained with anti-EGFR (red) and DAPI (grey). Minicells contain GFP (green).

16.5. Influence of bacterial flagella in the adhesion to tumor cells

The poor adhesion to tumor cells achieved by minicells prompted us to investigate which specific property present in live bacteria is responsible of their extreme efficient adhesion capacity. There are many important differences between minicells and the parental live bacteria, including size (four times smaller, size of minicells; 400nM)(MacDiarmid et al., 2007), lack of chromosomal DNA, and of an active metabolism and flagella. In the video number 1 shows an in vitro adhesion assay performed with WT strain EcM1 SAegfr2 and the tumor cell line Her14, in which motile bacteria are seen touching different points of the cells before establishing a permanent adhesion event. These type of microscopic observations suggested that the chance of finding a receptor might be increased by the flagellar motility, which doesn't not exist in the minicells. Flagella is the principal motility organelle in *E.coli* and other bacteria and it has been shown to be important for the adhesion of pathogenic bacteria to host cells (Duan et al., 2013; Haiko and Westerlund-Wikstrom, 2013) and and abiotic surfaces in biofilm formation (Belas, 2014; Friedlander et al., 2015). Based on these preceding reports and observations, we decided to focus on flagella and its role during the adhesion of our engineered bacteria to target tumor cells.

5.1. Adhesion of flagellar mutant bacteria to tumor cells

We constructed several mutant strains having deletions in genes related with the synthesis of flagella. First, we recreated in *EcM1SAegfr* strain the *ΔfliCD* mutation found in the E. coli strains producing the minicells. The *fliCD* genes encode the flagellin (FliC) and the flagellar cap protein (FliD) (Inaba et al., 2013). We tested the adhesion capability of the parental "WT" and *ΔfliCD* strains to Her14 cells (EGFR+). Bacteria were added to Her14 cells for 30 minutes (MOI 300:1), washed with PBS and stained for fluorescence microscopy (Figure 30).

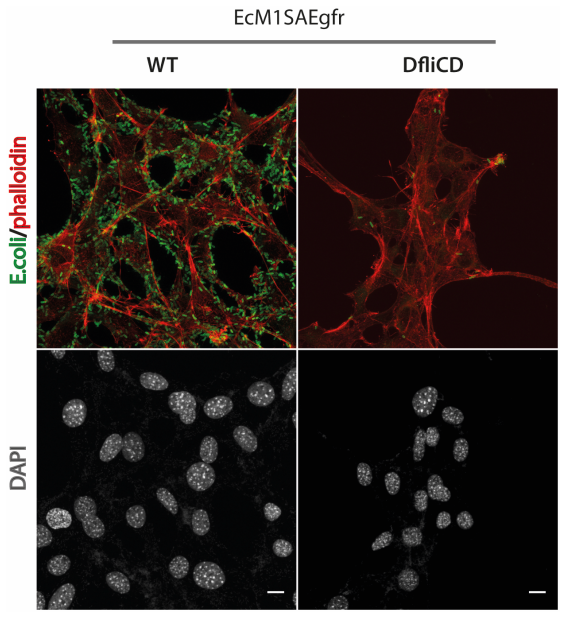


Figure 30. Adhesion to EGFR+ cells of engineered bacteria with wild type and mutant flagella. Confocal microscopy images of Her14 cells infected with EcM1luxSAegfr (WT) and isogenic mutant ∆isoge. Infection and staining actin (red) E.coli contains GFP under control of Ptac promoter for visualization in green. Cell nuclei and bacterial chromosomes were stained with DAPI (grey). Scale bar = 10 µm.

This experiment clearly shows that bacteria lacking flagella are not able to attach to Her 14 cells while motile bacteria adhere in high numbers. This result points out flagella as an essential piece in the adhesion process.

Although the flagellum has been traditionally regarded as a motility organelle, it has also been suggested to have an adhesin role in various pathogenic strains of *E. coli*, *Pseudomonas aeruginosa* and *Clostridium difficile* (Duan et al., 2013; Giron et al., 2002).

The role of flagella as an adhesin assisting the attachment of bacteria harboring SAs against EGFR was tested by constructing deletion mutants lacking the whole flagellar apparatus (∆*flhDC*) and abrogating motility (∆*motA*). The *flhDC* operon encodes a master regulator required for the expression of all the flagellar components (Gauger et al., 2007; Liu and Matsumura, 1994). The motAB operon encodes integral inner membrane proteins that form the stator of the flagellar motor, thus ∆*motA* mutants express flagellar filaments unable to rotate (Blair and Berg, 1990; Hosking et al., 2006).

The motility of the parental *EcM1SAegfr* (WT, positive control) and its deletion mutant's ∆*motA* and ∆*flhDC* strains was evaluated in soft LB-agar plates. As a negative control this assay also contained the non-motile EcM1∆*fliCD* strain. As expected, only the WT strain was able to swim in soft agar (Figure 31A). Coomassie staining of extracellular proteins from culture supernatants of these strains (Figure 31B) confirmed the absence of flagellar proteins FliC and FliD in the deletion mutants ∆*fliCD* and ∆*flhDC*, whereas these filament components are present in both the non-motile ∆*motA* mutant and the parental (WT) strain. We also showed that these deletions did not affect the expression of the synthetic adhesin on the bacterial surface (Figure 32A.), or the recognition of the EGFR (Figure 32B).

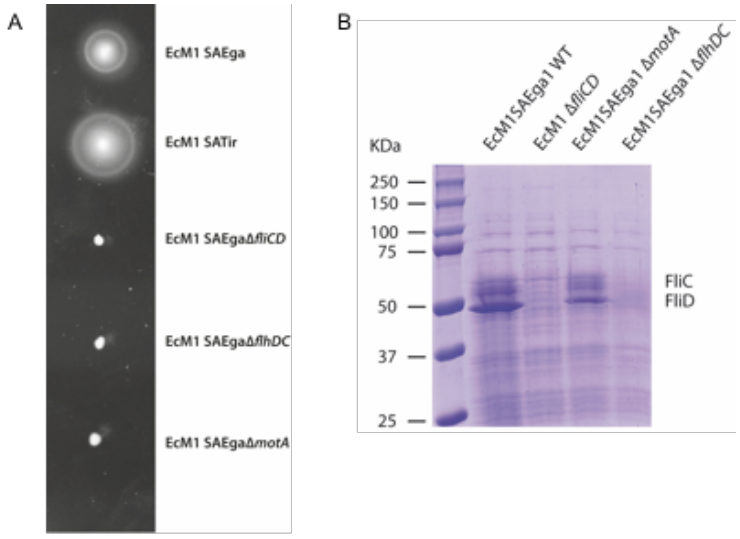


Figure 31. **A**. Motility assay on soft-agar plates of EcM1SAegfr strain (WT) and its mutant derivatives Δ*motA* and Δ*flhDC*. Bacteria were grown in a soft-agar (0.3 %) LB plate for 8 hours at 37°C. **B** Coomassie staining of SDS-PAGE (12%) having protein samples from culture supernants of the strains EcM1SAegfr (WT) and isogenic mutants Δ*motA* and Δ*flhDC*. EcM1Δ*fliCD* was used as a non-motile control (A and B).

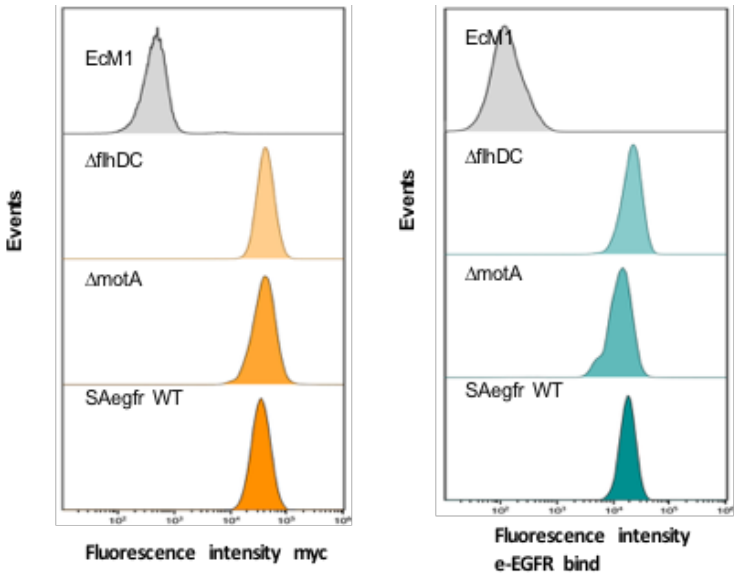


Figure 32. Surface display levels and functionality of SAs expressed in flagellar mutants of *E. coli*. Flow cytometry analysis of E. coli EcM1 (negative control; grey), EcM1SAegfr (WT), and isogenic Δ*motA* and Δ*flhDC* bacteria. Histograms shows the fluorescence intensity of bacteria stained with **A**. anti-myc mAb and secondary anti-mouse IgG-Alexa 488. **B.** biotinylated eEGFR-Fc and the secondary Streptavidin-APC.

Next, we tested adhesion of the above bacterial strains to Her14 cells (EGFR+). As a negative control of binding we used a flagellated WT strain expressing an unrelated SA (EcM1SAtir). Our results show that only the motile WT bacteria expressing SAegfr adhere to Her14 cells (Figure 33) which underlines the requirement of an active flagellar motor for the adhesion of E. coli to tumor cells.

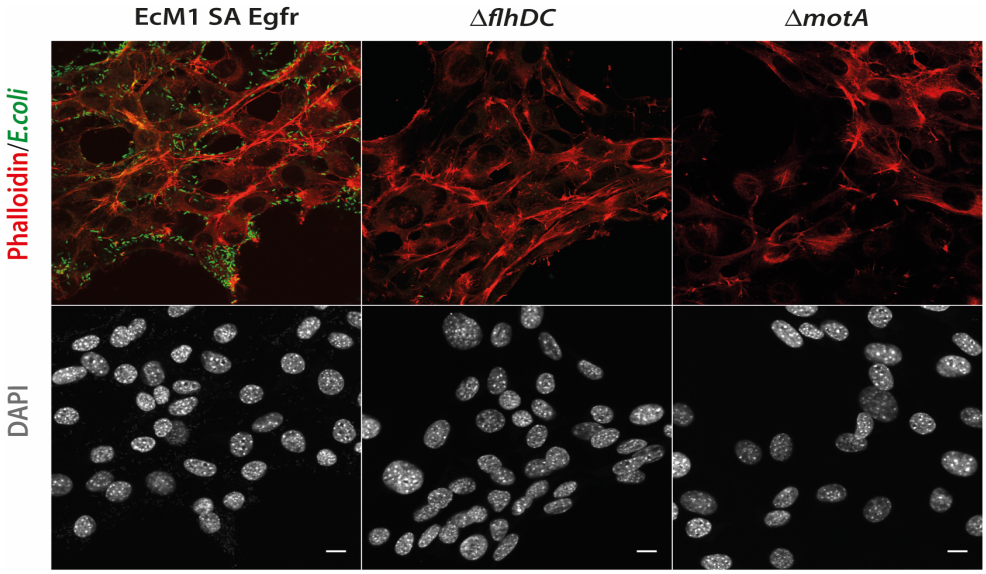


Figure 33. Adhesion of bacteria to target tumor cells requires an active flagellar motor. Confocal fluorescence microscopy images of the adhesion to Her14 (EGFR+) cells of *E. coli* strains EcM1SAegfr and isogenic Δ*flhDC* and Δ*motA* mutants. Cells were infected with bacteria (MOI 300:1) for 30 min and washed with PBS to remove unbound bacteria. Samples were stained to visualize actin (red), and DAPI (grey). Bacteria (green) express GFP from plasmid pGEN22 (REF). Scale bars =10 μm.

5.2 Importance of the sense of flagellar rotation for bacterial adhesion

The flagella motor rotates both clockwise (CW) and counterclockwise (CCW), which regulates the swimming pattern of bacteria (Xie et al., 2011). When all flagellar motors spin CCW, the bacterium presents all flagellar filaments in a single bundle a linear swimming occurs (run mode). In contrast, when the motors spin CW, the flagellar filaments spread in opposite directions and make the bacterium to tumble (tumble mode). Tumbling allows the bacterium to change its swiming direction randomly when reenters in the run mode. The frequency and time of CCW and CW rotation of flagellar motors is regulated in response to environmental stimuli generating positive chemotaxis to attractants and negative chemotaxis to repellents (Terashima et al., 2008). Both modes of bacterial swimming are necessary for the movement of bacteria towards environments that are favorable for growth and survival (chemotaxis), but it has not been described if these movements are important for bacterial adhesion to host cells.

Hence, we generated two deletion mutants from the *EcM1SAegfr* strain obtaining chemotactic mutant bacteria that only swims in a linear direction (Δ*cheY)* and bacteria that only tumble (ΔcheZ). The *cheY* and *cheZ* genes belongs to the chemotaxis regulators of *E. coli*, which are in charge of controlling the sense and duration of

rotation of flagellar motors (Belas, 2014). As a result of the presence of a repellent, CheY is phosphorylated (CheY~P) by specific receptors and interacts with the flagellar motor promoting the tumbling movement. CheY~P is dephosphorylated by CheZ and the linear swimming is restored (Akhter and Amin, 2017). We showed that in liquid LB medium, EcM1SAegfr Δ*cheY* and Δ*cheZ* strains have the expected phenotypes (see videos 2 and 3), with cheY deletion mutant only swimmin g linearly and the *cheZ* deletion mutant only tumbling. Also as expected, the *cheY* and *cheZ* mutants from *EcM1SAegfr* strain were non-motile in soft-agar plates (Figure 34), which confirms the absence of chemotactic movement in both mutants. In addition, we confirmed normal surface display levels and binding activity of SAegfr on Δ*cheY* and Δ*cheZ* mutants (Figure 34)

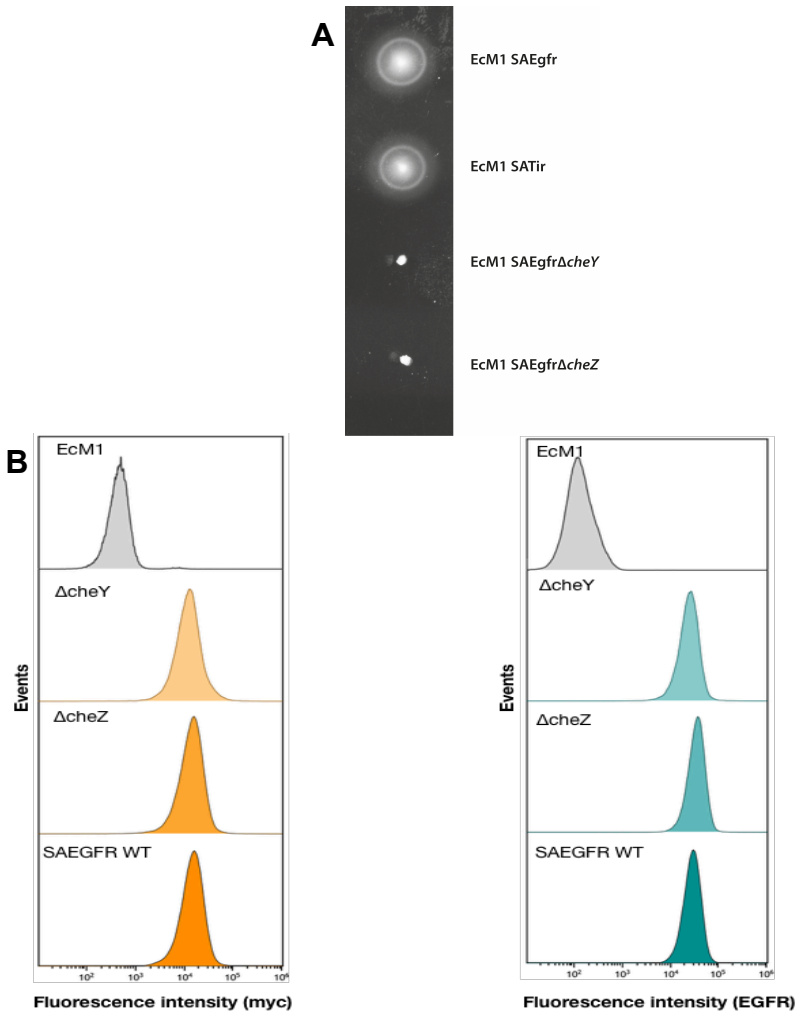


Figure 34. **A.** Motility and synthetic adhesin expression of chemotaxis mutants. Motility assay on soft-agar plates of EcM1SAegfr strain (WT) and its mutant derivatives ΔcheY and ΔcheZ. Bacteria were grown in a soft-agar LB plate for 8 hours at 37°C. Wild-type motility of EcM1SAtir strain is also shown. **B.** Surface display levels and functionality of SAs expressed in chemotaxis mutants of E. coli. Flow cytometry analysis of E. coli EcM1 (negative control; grey), EcM1SAegfr

(WT), and isogenic ΔcheY and ΔcheZ bacteria. Histograms shows the fluorescence intensity of bacteria stained with anti-myc mAb and secondary anti-mouse IgG-Alexa 488 (left pannel) and biotinylated eEGFR-Fc and the secondary Streptavidin-APC (right).

We tested adhesion of chemotaxis mutants to Her14 (EGFR+) cells and found that ΔcheY bacteria were able to bind to cell surface whereas ΔcheZ bacteria could not 35 This result suggested that a linear movement of the bacterium was needed for adhesion to the tumor cell surface. Microscopic inspection of these samples allowed us to conclude that bacteria lacking linear swimming were not able to reach the bottom of the well, where tumor cells are attached to the plastic (see video nº 2).

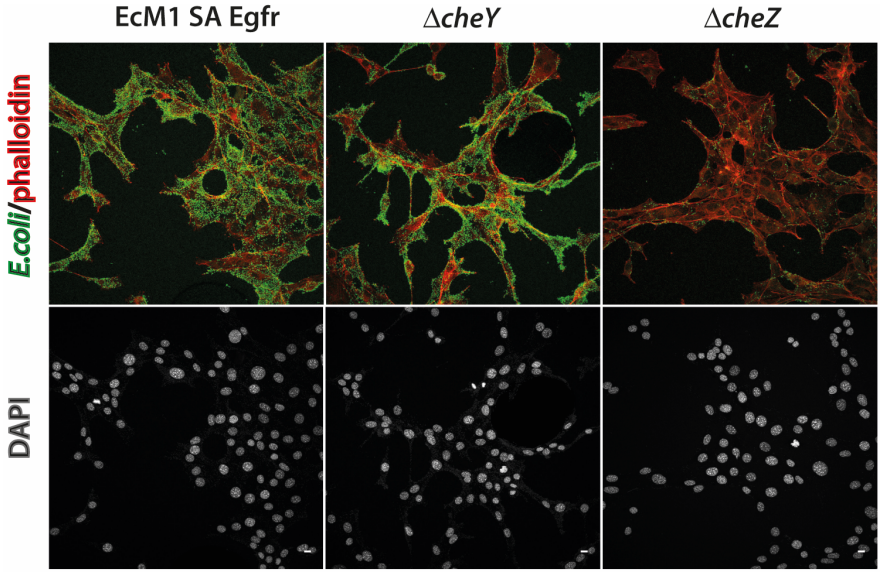


Figure 35. Adhesion of chemotaxis *E. coli* mutants to target tumor cells. Confocal fluorescence microscopy images of the adhesion to Her14 (EGFR+) cells of *E. coli* strains *EcM1SAegfr* and isogenic Δ*cheY* and Δ*cheZ* mutants..Samples were infected and stained as in Figure 25. Actin (red), bacteria (green) and cell nuclei (grey). Scale bars =10 μm.

To force the contact of bacteria to the tumor cells, we performed adhesion assays in which bacteria and cells were centrifuged at low speed on the culture plate (4000 rpm, 2 min), thus allowing bacteria to reach the bottom of the plates. In this experimental setup, the ΔcheZ mutant was able to bind the Her14 cells at similar levels as the WT and ΔcheY bacteria (Figure 36). Forcing cell contact by centrifugation of ΔflhDC and ΔmotA strains was insufficient to fully restore adhesion (Figure 36). Although some improvement was observed in these strains, the background adhesion of the control strain (EcM1SAtir) was also significantly higher. Therefore, the flagellar

rotation in at least one direction (CCW or CW) is required for an efficient adhesion of bacteria upon cell contact.

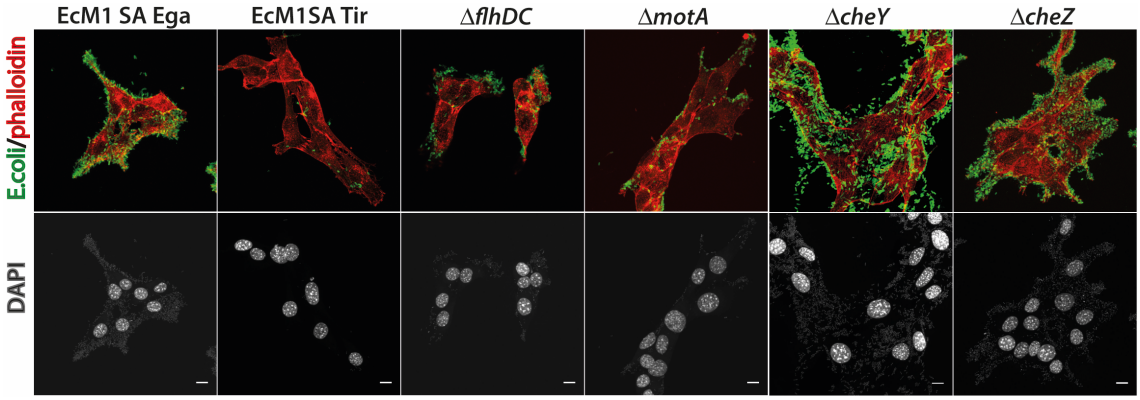


Figure 36. Forcing cell contact by centrifugation does not substitute the need of flagellar rotation. confocal microscopy images of Her 14 cells infected with EcM1SAtir (negative control), EcM1SAegfr (WT flagella), and isogenic ΔcheY, ΔcheZ, ΔflhDC, and ΔmotA bacteria. Bacteria and cells were centrifuged (2000 rpm, 4 min). and incubated for additional 30 min at 37 oC. Samples were stained to visualize actin (red) and cell nuclei (grey). Bacteria (green) express GFP in their cytoplasm. Scale bars=10 μm.

5.3 Adhesion assays under flow conditions to force cell contact

We explored the possibility of restoring the attachemnt to cells of ΔflhDC and ΔmotA mutants using an adhesion assay under unidirectional flow conditions, which could mimick the unidirectional swimming of ΔcheY bacteria. For these experiments, Her14 cells were grown on slides with microchannels (IBIDI channel μ-slides VI 0.4) and bacteria were passed in media at two different flow rates (Figure 29 nueva). A flow rate of 20 μl/min, corresponding to a shear stress of 0.025 dyn/cm^2, and a flow rate of 100 μl/min, corresponding to a shear stress of 0.126 dyn/cm^2 . The shear stress (τ) was calculated according to the formula $\tau=\eta\bullet 176.1\bullet\phi$ for this type of chamber h is the dynamical viscosity of the media (0.0072 dyn·s/cm^2 for DMEM with 10% of serum at 37°C) and f the flow rate).

Bacteria were passed for 30 min and then the channels were washed with media for additional 5 min at identical flow rate. In both cases, we found that WT bacteria (EcM1SAegfr) adhered to Her14 cells whereas ΔmotA and the ΔflhDC strains were not able to attach under any condition (Figure 30 nueva A and B). These phenotypes are observed in videos 4, 5, and 6. Taken together, these results strongly indicate an important role of the activity of the flagellar rotor for bacterial adhesion upon cell contact, which cannot be imitated by centrifugation or unidirectional flow.

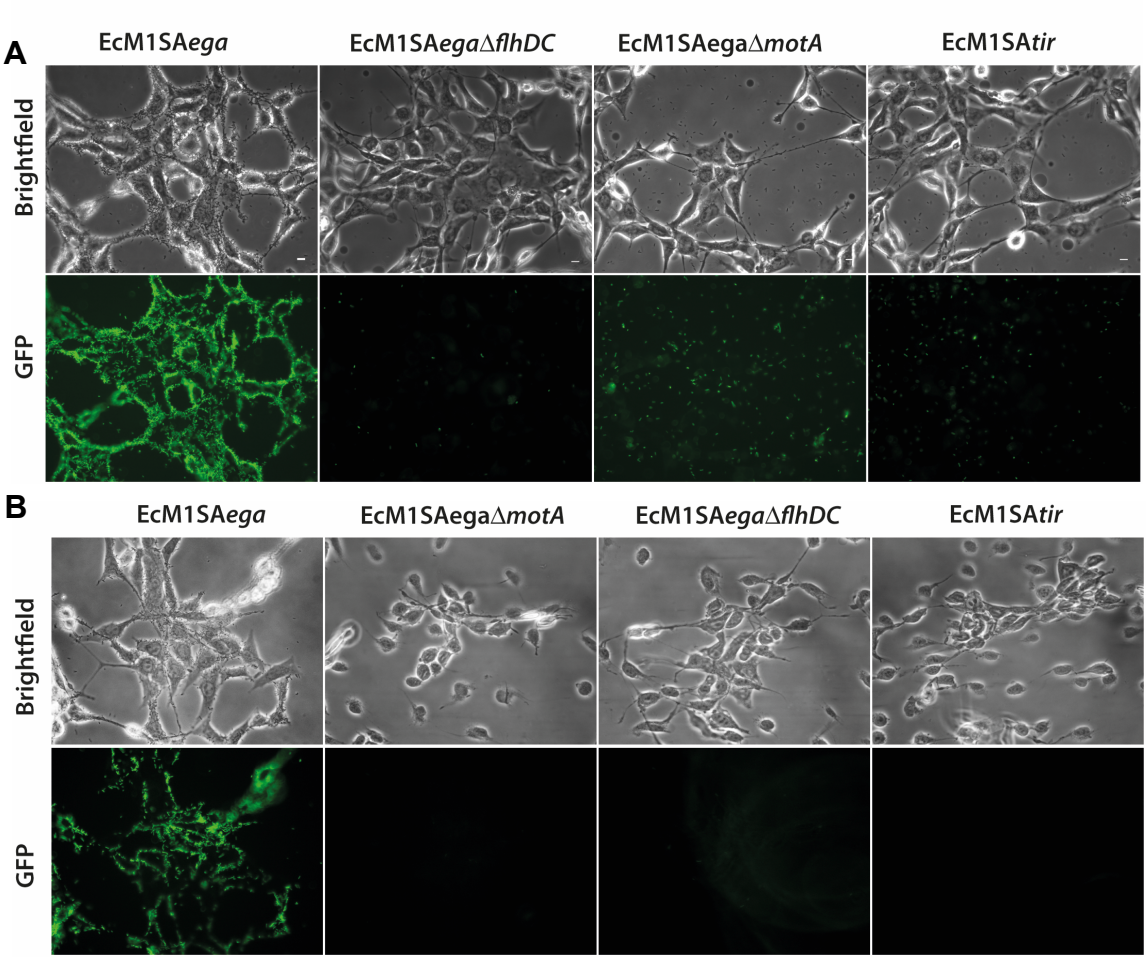


Figure 37. Adhesion assays under unidirectional flow conditions. Bright field and fluorescence microscopy images of Her14 cells infected with EcM1SAtir (negative control), EcM1SAegfr (wild-type flagella), and isogenic ΔflhDC, and ΔmotA bacteria (as indicated). Bacteria express GFP (green) and were applied for 30 min with media at a flow rate of 20 µl/min (A) or 100 µl/min (B). Scale bar =10µm.

5.4 Evaluating flagellar mechanosensing in bacterial adhesion.

We hypothesized that that flagellar rotation could trigger a signaling cascade in the bacterium that may facilitate the attachment to the tumor cell surface. It has been reported that mechanosensing of surfaces in bacteria is a process that often uses obstruction of flagellum rotation to trigger behaviors such as adhesion and surface-associated movement (Ellison and Brun, 2015). In *E. coli*, an outer membrane lipoprotein, called NlpE (Delhaye et al., 2019), is reported to be able to sense a mechanical contact to a solid surface and activate a poorly characterized signalling pathway to pause the flagellar motor . This results in an increase in c-di-GMP levels in the bacteria cytoplasm, which activate a flagellar "brake" protein called YcgR (Boehm

et al., 2010; Lacanna et al., 2016). A major enzyme involved in c-di-GMP production in E. coli is DgcZ (Lacanna et al., 2016).

To explore whether this mechanosensing pathway participates in the adhesion process to cells, we generated deletion mutant strains ΔnlpE, ΔycgR, and ΔdgcZ derived from our motile wild type *EcM1SAegfr* strain. These mutant strains were motile in soft agar at the same level as *EcM1SAegfr* (Figure 38) and expressed functional synthetic adhesins on the bacterial surface (Figure 38). We also confirmed the expected phenotype of ΔycgR by overproducing in this mutant the diguanylate cyclase enzyme AdrA from *Salmonella* enterica sr. typhimurium (Cowles et al., 2016). Overxpression of AdrA increase the level of di-c-GMP and stops rotation of flagella by interaction with YcgR (Zorraquino et al., 2013). Induction with AdrA expression from plasmid pBADadrA inhibits the motility of the wild type EcM1SAegfr but not of the ΔycgR (Figure 39).

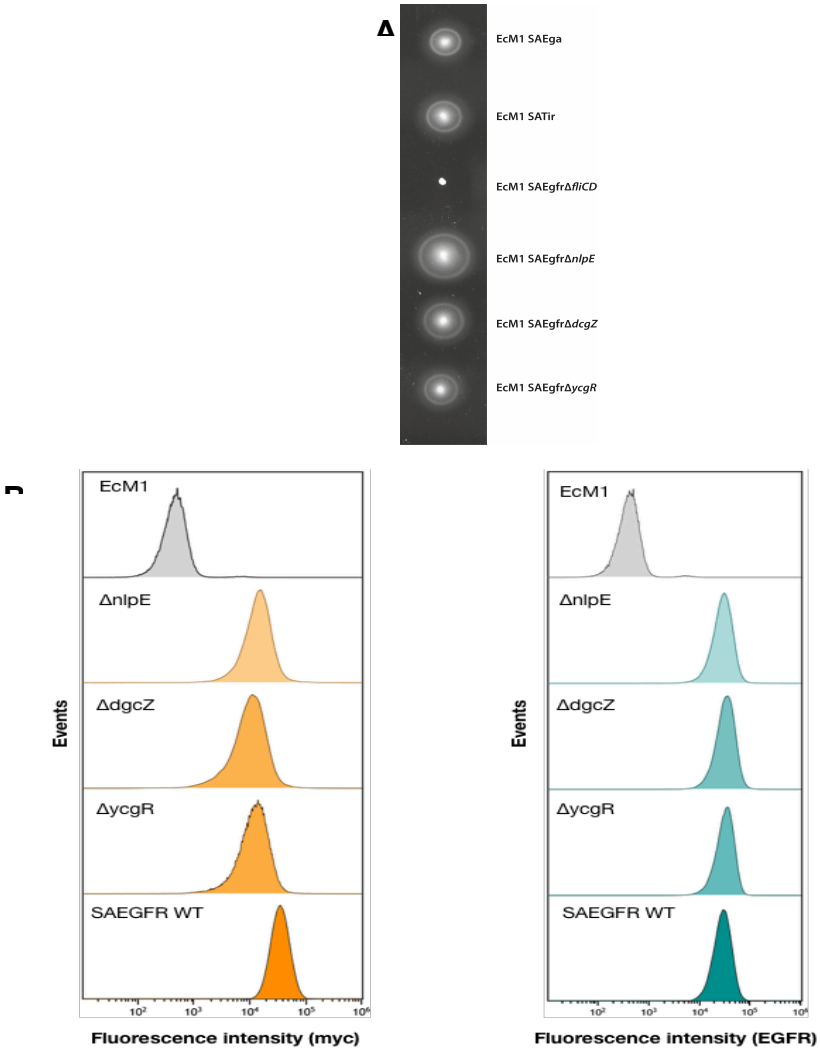


Figure 38. Motility and synthetic adhesin expression of flagellar mechanosensing mutants. **A**. Motility assay on soft-agar plates of EcM1SAegfr strain (WT) and its mutant derivatives ΔnlpE, ΔycgR, and ΔdgcZ. Bacteria were grown in a soft-agar LB plate for 8 hours at 37°C. Wild-type motility of EcM1SAtir strain is also shown. **B**. Surface display levels and functionality of SAs expressed in mechanosensing mutants of *E. coli*. Flow cytometry analysis of E. coli EcM1 (negative control; grey), EcM1SAegfr (WT), and isogenic Δ*nlpE*, Δ *pE1*, and Δ*dgcZ* bacteria. Histograms shows the fluorescence intensity of bacteria stained with anti-myc mAb and secondary anti-mouse IgG-Alexa 488 (left pannel) and biotinylated eEGFR-Fc and the secondary Streptavidin-APC (right).

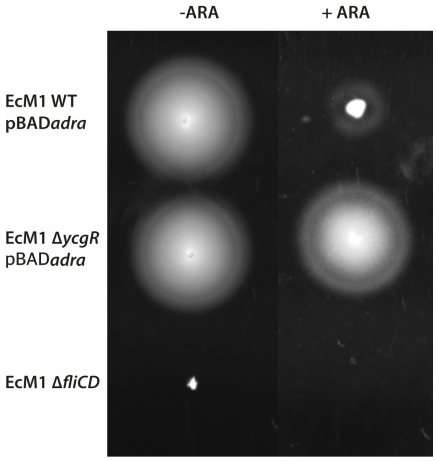


Figure 39. Inhibtion of flagellar motility by di-c-GMP production is dependent on YcgR. Motility in sofr-agar plates of EcM1SAegfr and ΔycgR strains carrying pBADadrA grown in the absence (-) or presence (+) of L-arabinose (Ara). The strain EcM1ΔfliCD is included as a non-motile control.

Next, we performed an standard in vitro adhesion assay to cells and found that ΔnlpE, ΔycgR, and ΔdgcZ deletion mutants attached to Her14 cells at the same level that the parental strain EcM1SAegfr (Figure 33).

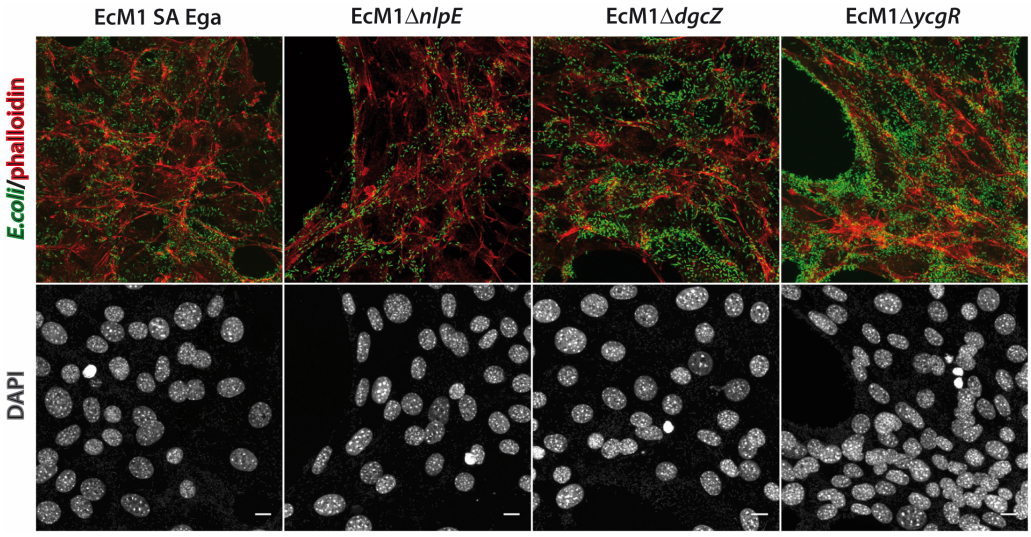


Figure 40. Adhesion of mechanosensing *E. coli* mutants to target tumor cells. Confocal fluorescence microscopy images of the adhesion to Her14 (EGFR+) cells of *E. coli* strains EcM1SAegfr and isogenic Δ*nlpE*, Δ*ycgR*, and Δ*dgcZ*. Samples were infected and stained as in Figure 25. Actin (red), bacteria (green) and cell nuclei (grey). Scale bars =10 μm.

5.5 Changes in cytoplasmic membrane potential during bacterial adhesion.

It has been reported that the sudden stop of flagellar rotation upon contact to a solid surface leads to an increase in the electrochemical potential of the cytoplasmic membrane ($\Delta\Psi$) due to the blocking of the proton flux through the flagellar motor (Van Dellen et al., 2008). This rapid increase the concentration of protons in the periplasm may neutralize negative charges in the bacterial OM, thus facilitating adhesion of bacteria to surfaces with net negative charge (Van Dellen et al., 2008). Interestingly, the surface mammalian cells, and especially in the case of tumor cells, is reported to have a net negative charge. Some studies have also reported that a net positive charge of nanoparticles (Rahman et al., 2017) and bacteria (Heckels et al., 1976) enhance their attachment to mammalian cells.

To evaluate the role of $\Delta\Psi$ we examined the effect of valinomycin in the bacterial adhesion to Her14 cells. Valinomycin is a neutral ionophore for cations, with a high specificity for K+, that permeates biological membranes freely dissipating ion gradients across the membrane and the $\Delta\Psi$. Valinomycin combines reversibly with K^+ ions to form a membrane-permeable complex that diffuses across the inner membrane and releases K^+ on the inside. This movement of charge reduces the value of $\Delta\Psi$ but does not affect motility of flagella. However, we found that addition of Valinomycin during the adhesion assay does not interfere with the attachment of the *EcM1 SAegfr* bacteria to Her14 cells. Therefore, the transient increase of $\Delta\Psi$ induced by the stop of flagellar motors upon contact with a surface is not required the adhesion of engineered bacteria to tumor cells.

Flagellar rotation is needed for establising multiple contacts of bacteria with the cell surface.

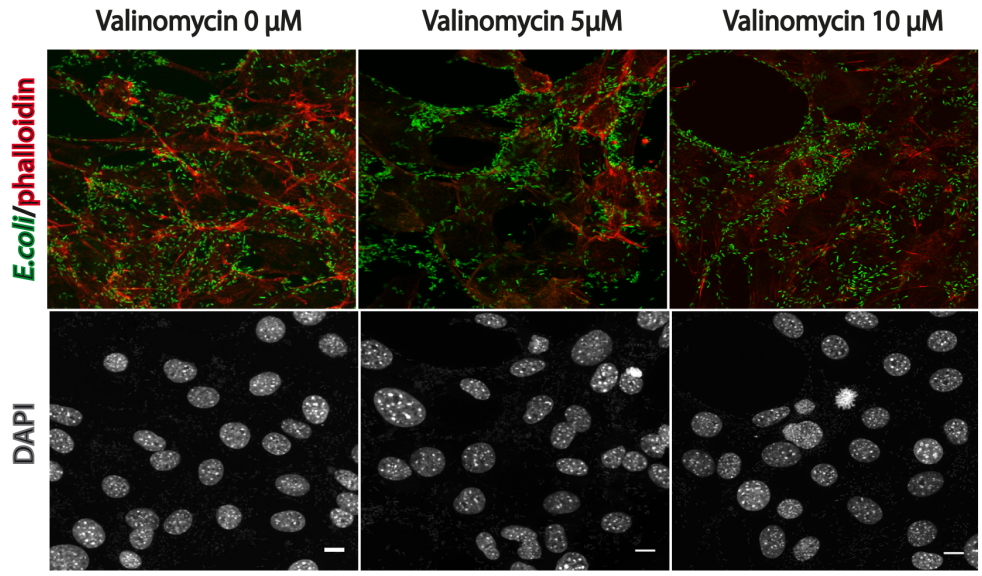


Figure 42. Inhibiting the electrochemical membrane potential does not inhibit bacterial adhesion to target tumor cells. Confocal fluorescence microscopy images of Her14 cells infected with EcM1SAegfr bacteria incubated with Valinomycin at the indicated concentrations (0, 5 and 10 µM). Samples were stained to visualize actin (red) and cell nuclei (grey). Bacteria (green) express GFP in their cytoplasm. Scale bars=10 µm.

Albeit our previous data recognized the importance of flagellar rotation for adhesion, we did not have a satisfactory model that could explain how flagella act during the attachment of bacteria to cells. To gain some insight into this issue, we performed detailed live video microscopy with the idea to observe the adhesion process in real-time. (See videos V7 and V8). In the vas majority of cases we observed that motile bacteria reach the surface of the mammalian cell and "scan" briefly the cell surface before establising or not a permanent adhesion. These initial contacts are mediated by one pole of the bacterium, opposite to the direction of flagellar filaments and bacterial movement (See Figure 43A and video 7). The bacterium may stay in this "transient" adhesion stage for ca. 1 min, twisting around this intial contact point. If no additional contacts with the cell surface are established during this stage, the bacterium eventually leaves swimming in a different direction and then permanent adhesion does not occur. In contrast, if the bacterium is able to find an additional anchor point in the cell membrane, and thus more than one adhesion contacts are established simultaneously, the initial transient adhesion transforms into a a permanent attachment of the bacterium (Figure 43B and video 8). In these cases, the bacterium rod is parallelel to the cell surface and contacts are established along the bacterial body and not in only at the bacterial pole. Eventually, bacterium with a lateral contact to the cell stops any visible movement, suggesting that the flagellar motors have stopped.

Therefore, these data indicate that a motile flagella is needed for the bacterial twisting after the initial contact in order to establish additional contacts that transform the transient contact into a permanent attachment.

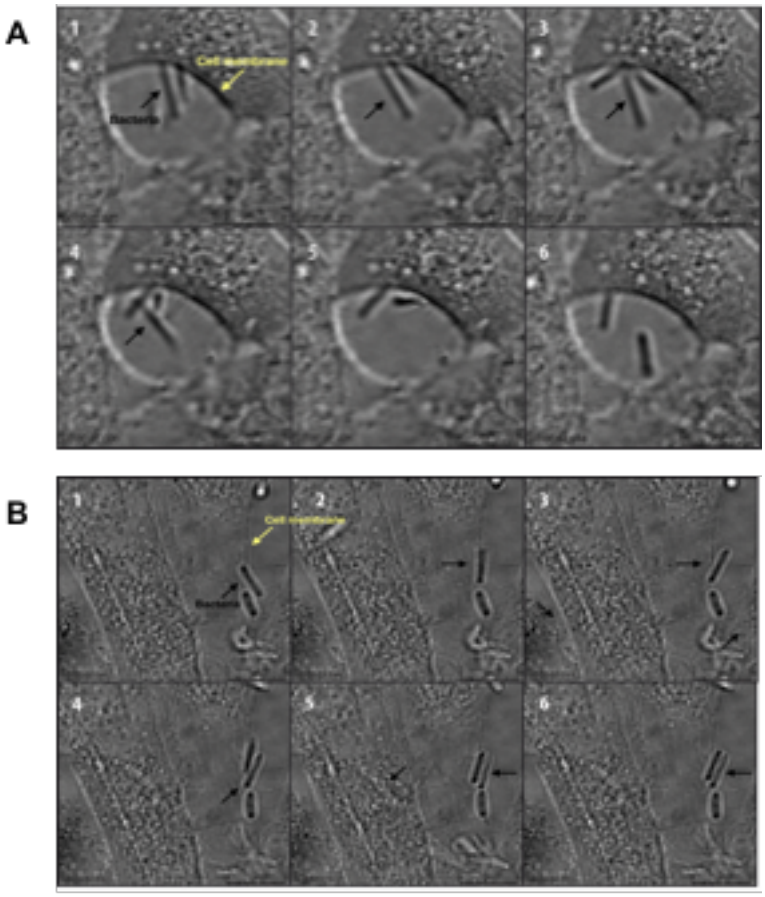


Figure 43. Live microscopy images of the bacterial adhesion process. Bright field microscopy images of six time points (within 2 min) showing a representative example of a bacterium (pointed with an arrow) contacting a cell with the bacterial pole and **(A)** not producing a permanent adhesion or **(B)** leading to a permanent adhesion establishing multiple contacts with the bacterial body. Her14 cells were infected with EcM1SAegfr bacteria at a MOI 30:1.

5,6 Requirement of flagella for bacterial cell attachment mediated by natural adhesins

At this stage, it was interesting to investigate if the dependence on the flagellar motility also happens with natural adhesins expressed by pathogenic bacteria for host cell attachment, and not only in the engineered E. coli strains with synthetic adhesins. Interestingly, it has been reported that the absence of flagella in certain pathogens reduces significantly their virulence (Chaban et al., 2015). To investigate the role of flagella for cell attachment mediated by pathogens, we expressed in *E. coli* different adhesins from pathogenic strains and performed adhesion assays to mammalian cells grown in culture.

We first expressed invasin form Yersinia pseudotuberculosis given its structural similarity to synthetic adhesins (Oberhettinger et al., 2012). Invasin is an OM adhesin closely related with intimin from EPEC and EHEC strains. The structure is composed of an N-terminal signal peptide, a short periplasmic α domain, a β-barrel domain inserted in the OM, and a repeat of extracellular Ig-like (D1-D4) domains and a C-terminal lectin-like domain. Invasin binds β-integrins on the host cell surface to trigger internalization of the bacteria.

We expressed invasin under the control of the P_{BAD} promoter, using the suicide vector employed previously for invasin integration in yfaL (Figure 9). This construct was integrated in EcM1 wild type and Δ*fliCD* strains expressing GFP. Bacterial cultures of the resulting strains were induced with L-Ara and used to infect Her14 cells for 30 min. After washing unbound bacteria, samples were stained for fluorescence microscopy (Figure 44). This experiment showed that bacterial adhesion mediated by Invasin also requires the expression of flagella.

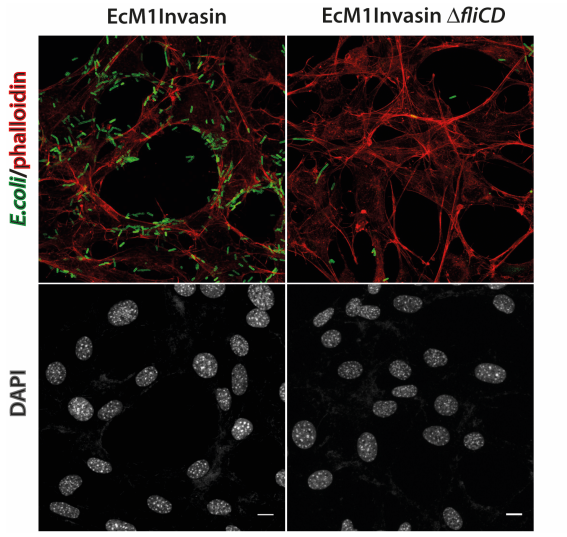


Figure 44. Invasin-mediated cell adhesion requires expression of flagella in E. coli Confocal microscopy images of Her14 cells infected for 30 min with E. coli wild type and ΔfliCD strains expressing invasin and GFP. Strains EcM1-araCPBAD-inv and EcM1ΔfliCD-araCPBAD-inv were grown with L-Ara and used to infect Her14 cells for 30 min (MOI 300:1). Samples were infected and stained as in Figure 25. Actin (red), bacteria (green) and cell nuclei (grey). Scale bars =10 μm.

Next, we decided to test a natural adhesin with a completely different structure, namely the type I fimbriae, which are a crucial factor for the infection of uropathogenic E.coli (UPEC) mediating the adhesion of bacteria to manose-containing glycoproteins of human urothelium (Terlizzi et al., 2017). The adhesin tip subunit of type I fimbria (FimH) consist of an N-terminal lectin domain with the mannose-binding site and a C-terminal pilin domain responsible for incorporating FimH into the fimbrial structure

(Mydock-McGrane et al., 2017). The EcM1 strain, which has a deletion in the chromosomal type I fimbria operon (fimA-H), and its isogenic non-motile strain EcM1ΔfliCD, were transformed with plasmid pSH2, encoding the entire fim operon from UPEC strain J96 (Hagberg et al., 1983). In vitro adhesion assays using these bacteria on the human bladder cell line HTB9 showed that only motile wild type bacteria expressing the type I fimbria attached to human bladder cells HTB9 (Figure 45).

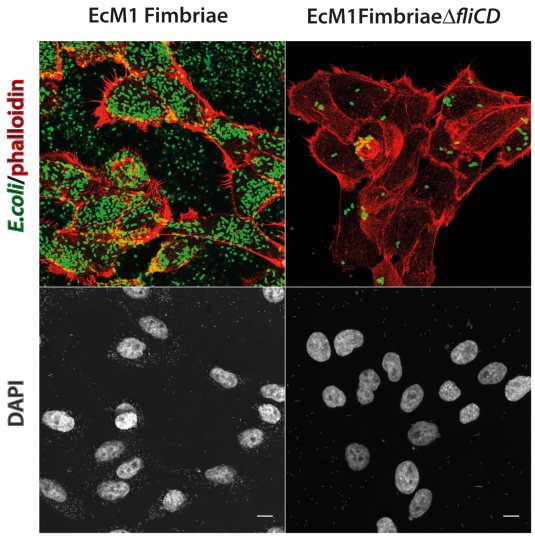


Figure 45. Type I fimbriae-mediated adhesion of human bladder cells requires expression of flagella. Confocal microscopy images of HTB-9 human bladder cells infected for 30 min with EcM1 motile and ΔfliCD mutant bacteria (MOI 300:1) expressing GFP and carrying pSH2, encoding the type I fimbriae operon from UPEC J96 strain. Samples were infected and stained as in Figure 25. Actin (red), bacteria (green) and cell nuclei (grey). Scale bars =10 μm.

The above experiments demonstrate that flagella is an essential organelle for the adhesion of E.coli to mammalian cells, not only with synthetic adheins but also with natural afimbrial and fimbrial adhesins from pathogenic bacteria, like invasin and type I fimbria.

Lastly, we wanted to test whether flagella were also relevant for the adhesion of a non-E. coli pathogen like Salmonella enterica sv. typhimurium. It has been reported that the flagella is an important virulence factor for this strain, and this could be directly related with its ability of attach to the host cells (Barbosa et al., 2017).

Motile S. typhi sv.typhimurium strain and its isogenic non-motile ΔflhDC strain were used for infection of normal rat kidney (NRK) fibroblasts (MOI 100:1). After 1 h infection, cells were washed and differentially stained to visualize intracellular and extracellular bacteria by fluorescence microscopy (Figure 46). This experiment also revealed that attachment of S. typhimurium to host cells also depends on the expression of flagella, corroborating our previous results with E. coli bacteria.

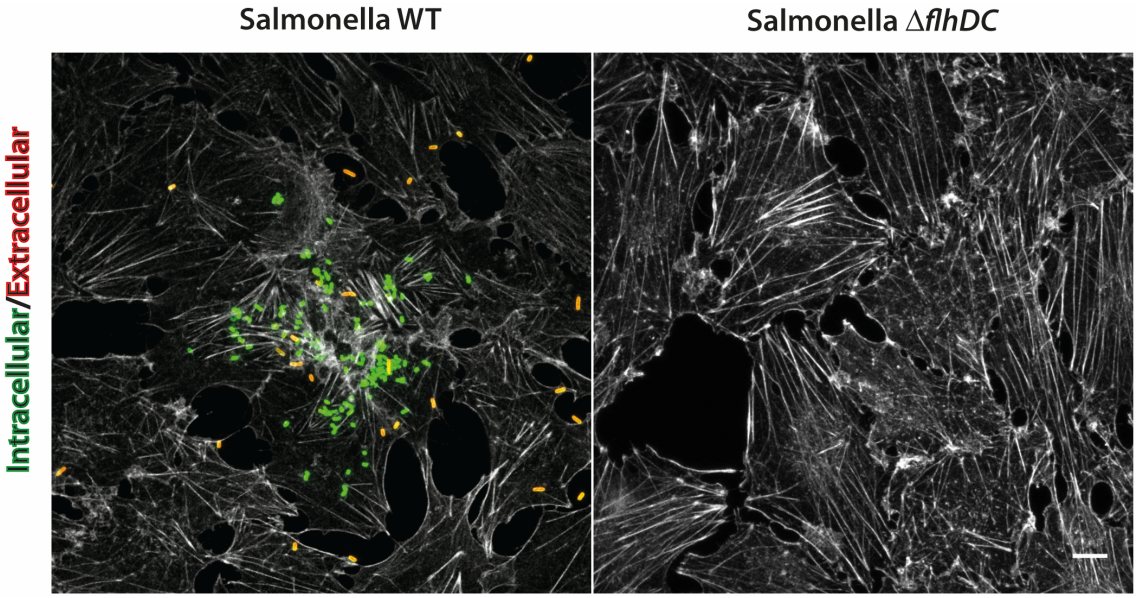


Figure 46. Adhesion of Salmonella enterica sv. typhimurium to NRK fibroblasts depends on the expression of flagella. Confocal microscopy images of NRK fibroblasts infected with S. typhimurium and isogenic ΔflhDC mutant bacteria (MOI 100:1) for 60 min. Extracellular bacteria were first stained with anti-Salmonella LPS rabbit polyclonal serum and secondary anti-rabbit-Alexa 594 (red). Cells were then permeabilized (0.1% TX-100) and stained with anti-Salmonella LPS and secondary anti-rabbit-Alexa 448 (green) to label both extracellular and intracellular bacteria. Extracellular bacteria are stained brown (red + green). Intracelllular bacteria are stained green. Cell nuclei and bacterial DNA were stained with DAPI (grey). Scale bars = 10 μm.

Discussion

1. Engineered bacteria targeting human epithelial cancer cells

Certain types of bacteria are extraordinary alternatives for treating cancer given their natural ability to colonize solid tumors, intratumoral penetration capability, and easy of genetic manipulation. However, numerous challenges remain before these bacteria can be used in the clinic, including intrinsic bacterial toxicity, targeting efficiency and genetic instability. Several strategies have been developed for modifying bacteria with this purpose and the most widely studied bacteria for cancer therapy is S. typhimurium. But *Salmonella* presents obvious inconveniences given that its pathogenic character, immunogenicity and risk of causing a septic shock.

To reduce the immunogenicity of *Salmonella*, the VPN20009 strain was created by deleting both the purI and the msbB genes (Pawelek et al., 1997). This attenuated Salmonella strain has been used already in Phase I clinical trials involving patients with advanced or metastatic cancer where $3x10^8 CFU/m^2$ was defined as the maximum tolerated dose in 25 patients treated intravenously with the bacteria. Colonization of the tumor was observed in just three of patients and no tumor regression was observed (Cunningham and Nemunaitis, 2001). Some clinical toxicity was also reported like hypotension, fever, anemia, diarrhea, nausea, and vomiting, probably due to the high dose of bacteria (Cunningham and Nemunaitis, 2001). The reduction of the bacterial dose for tumor colonization and the use of a less immunogenic bacterial strain could definitely present an essential alternative for developing an anti-cancer therapy using bacteria.

Previously in our laboratory, a non-pathogenic *E. coli* K-12 strain lacking the Type 1 fimbriae, Antigen 43 and Mat fimbriae, were modified to express a SA targeting GFP antigen expressed on the surface of HeLa tumor cells. This experimental model allowed a significant reduction (two orders of magnitude) of the bacterial dose needed for optimal tumor colonization in vivo. In addition, it was found out that the engineered E.coli strain is retained at lower levels than the wild type strain in non targeted organs such as liver and spleen probably caused by the lack of some adhesins naturally expressed by *E. coli* (Pinero-Lambea et al., 2015a).

Based on this work, we aimed to develop SAs recognizing actual surface antigens expressed on human tumor cells and target engineered *E. coli* bacteria against these molecules.

In this work, we have characterized the affinity of Nbs binding human EGFR (Salema et al., 2016) whose expression is altered in many epithelial carcinomas and which is the target of many approved anti-cancer drugs (Tebbutt et al., 2013). We employed these Nbs to build high-affinity SAs that allow *E. coli* to attach to human bladder and colon tumor cell lines expressing EGFR. Albeit our engineered bacteria attach with good efficiency to all EGFR-expressing cell lines tested, the adhesion levels were higher in colon cancer cell lines (e.g. HCT116, DLD2) than in bladder cell lines (e.g. T24, UMUC3). This result was surprising because EGFR expression levels is similar in all these cell lines according to flow cytometry analysis, and thus this difference could be due a reduced accesibilty of this receptor or a mechanism of inhibition of bacterial attachment by some undefined property of the surface of bladder cells. Despite these differences, bacterial attachment was sufficiently high and specific in both bladder and colon carcinoma cells to open the possibility of targeting these tumors using bacterial administration routes (e.g. intravesical, oral) that are safer than systemic administration into the blood stream. In comparision with the currently approved anti-EGFR antibodies (e.g. Cetuximab, Panitumumab) (Martinelli et al., 2009), whose action is confined to the irrigated areas of the tumor, bacteria expressing anti-EGFR SAs have the potential to be used at safer doses and actively penetrate into the core of the tumor to spread the therapeutic effect.

17.2. Engineering invasive bacteria of target tumor cells for release of protein payloads.

The principal aim of bacterial cancer therapies is to perform an action in the tumor area for tumor cell killing (Forbes, 2010). For this purpose, the bacteria need to be able to deliver the desired molecule in the tumor area, ideally specifically in the tumor cell, to avoid toxicity effectos in healthy tissues. The invasion and subsequent release of a therapeutic protein payload inside the target tumor cell was an interesting alternative that we have investigated in combination with SAs. Our results demonstrate that combining the constitutive expression of SAs binding EGFR with the subsequent inducible expression of invasin allow massive internalization of EGFR-positive tumor cells by the engineered bacteria. Leaky expression of invasin from PBAD promoter in media lacking glucose is suffient to observe internalization of individual bacteria, although induction with L-ara triggers a massive internalization of bacterial clusters.

Intracellular bacterial pathogens possess several mechanisms of invading the host cells including a fast escape from the phagosome and the modification of the vacuole

bacteria contents to avoid death via phagosomal acidification (Pizarro-Cerda and Cossart, 2006). The *E. coli* K-12 strain does not have any of these complex mechanisms and for the delivery of therapeutical molecules inside target cells, we needed to include the expression of different genetic modules. An alternative that has been previously used for the delivery of several types of molecules inside mammalian cells is the expression in E. coli of invasin from *Yersinia pseudotuberculosis* on the bacterial surface in combination with the pore-forming LLO protein from *Listeria monocytogenes* (Higgins et al., 1999). This strategy was repoerted to trigger internalization of the bacterium and the release of bacterial content into the cell cytoplasm. For instance, antigens like ovoalbumin (OVA) have been released in dendritic cell cytoplasm using *E. coli* expressing cytoplasmic LLO. Dendritic cells or macrophages are professional phagocytic cells and are highly specialized in the engulfment and lysis of invading bacteria. Therefore, bacteria enter these phagocytic cells without the need of invasin. The phagocytic cell is then able to lyse the bacteria liberating their content inside the phagosome. The LLO in the bacterial cytoplasm is released and forms pores in the membrane of the phagosome, delivering the bacterial content into the host cell cytoplasm (Higgins et al., 1999; Radford et al., 2002).

An engineered auxotrophic for diaminopimelic acid (dap) and invasive E. coli was reported to as being able to mediate gene transfer into epithelial cells transferring plasmid DNA vectors (Fajac et al., 2004). However, the gene transfer efficiency was low and took at least three hours for bacteria to reach the phagosome compartment. In the case of the DNA vectors, trafficking into the nucleus of the target cell should take place (Castagliuolo et al., 2005; Fajac et al., 2004). An invasive *E. coli* encoding shRNA against CTNNB1 (catenin b-1) was reported to induce gene silencing in the intestinal epithelium and in human colon cancer xenografts in mice (Xiang et al., 2006). These results indicated that invasive *E. coli* is able to release nucleic acids into the cytoplasm of non-phagocytic cells. However, these studies did not report the release of a protein paylod. To investigate this possibility we used a dap auxotroph and invasive E.coli expressing LLO to release a reporter protein enzyme (ß-lactamase) into HCT116 colon epithelial tumor cells. The E. coli strain expressed LLO into the bacterial cytosol or in the periplasm of the bacterium to facilitate a faster release of the protein in the phagosome. Despite massive bacterial invasion, only a weak liberation of ß-lactamase into the cytoplasm of the tumor cells was detected after 24 h of infection with the dap auxotroph strain expressing the LLO in the cytosol. This results suggest that some intracellular protein release is possible under in vitro conditions (cell culture), but the system might not be efficient in vivo. As a control, we included the SIEC strain (*E.coli*

K-12 expressing the type III secretion system from EPEC) (Ruano-Gallego et al., 2015) that is able to inject ß-lactamase directly to the target cell cytoplasm. This control shows a much higher efficiency of protein injection into the cytosol, especially at short times after bacterial adhesion. Bacterial T3SS are highly versatible systems allowing translocation of different effectors to heterologous proteins into the cytosol of mammalian cells (REFS) (Galan et al., 2014; Portaliou et al., 2016). Nonetheless, constrains in the pore diameter of T3SS and limitations in the unfolding capacity of the proteins to be translocated restrict the actual number of proteins able to be injected by these molecular nanosyringes (Barison et al., 2013; Lee and Rietsch, 2015). it is knwon that effector proteins of T3SS unfold in the cytosol in order to pass through the cytosolic entrance of the injectisome (Akeda and Galan, 2005). Strongly folded proteins are not translocated through injectisomes (Dohlich et al., 2014), (Notti and Stebbins, 2016). To be able to deliver a wider spectrum of molecules having these limitations, an improvement of protein delivery trough cell invasion might be necessary. A critical step in the whole process is the inability of rapid release of the bacterial content from the phagosome of non-phagocytic cells.

An interesting alternative for a faster liberation of cargo molecules after internalization could be the combination of the two delivery strategies develop in our laboratory, using the T3SS injectisomes to facilitate the escape of bacterial content from the phagosome. Interestingly, it has been reported the efficient escape of invasive E. coli bacteria from phagosomes after internalization thanks to the pore formation in the phagosome membrane by T3SS (Du et al., 2016). Although not all T3SS are able to reproduce this phenotype, (the type III secretion system from Yersinia does not produce the liberation of the bacteria (Du et al., 2016)), it would be interesting to test whether the EPEC T3SS in SIEC is able to produce the liberation of the E. coli from the phagosome in combination with the invasin protein.

18.

19.3. Minicells with synthetic adhesins as nanoparticles for protein delivery in tumor cells.

Systemic administration of chemotherapeutic drugs causes a severe toxicity in healthy tissues. A deeply studied approach to reduce the toxicity of these therapeutical molecules is their encapsulation in nanovehicles to deliver the drug only in the tumor area. In this regard, liposomes and other nanoparticles are able to accumulate in the tumor area thanks to the leaky vasculature resulting from the deregulated nature of the tumor angiogenesis (Miao and Huang, 2015). Once inside the tumor, these

nanoparticles break down and release a high concentration of the drug, reducing the general toxicity but still with side effects such as skin toxicity (including hand-foot syndrome and mucositis).

Nanoparticles can be targeted to specifically recognize tumoral cells and thereby achiving higher concentrations of the drug in the tumor area and making the therapy safer. Nanoparticles targeting cell surface receptors overexpressed in tumor cells such as EGFR or Her2 have being developed (Flemming, 2007).

An improvement in drug encapsulation will be to generate a nanoparticle that is easier to produce, able to contain a wide spectrum of molecules, possesses the sufficient integrity to not leak the therapeutic molecule until confined to the tumor area and recognizing specifically the tumor cells and not the healthy tissues. Minicells (bacteria derived nanoparticles) present an extraordinary option as nanovehicles and has been used already in antitumoral treatments for systemic administration of a wide spectrum of molecules including chemotherapeutics, siRNA, or bacterial toxins against tumoral cells (MacDiarmid and Brahmbhatt, 2011). Minicells are able to carry a concentration from one to ten millions of molecules in comparison with liposomes that can carry only up to 10.000 molecules. The targeting of minicells with bispecific antibodies recognizing the minicell surface and specific molecules on tumor cell surface, improved the tumor targeting in a considerable way (MacDiarmid et al., 2007). Minicell production protocol consists in different step from minicells purification, minicells loading with the therapeutic molecule and several steps of incubation with antibodies if we want to create target minicells. Interesntly, a recent study have reported the therapeutic effect of VAX-IP - an *E. coli*-derived minicells lacking dapA (DAP auxotrophy) and *lpxM* (less immunogenic lipid A) genes (for biosafety reasons) and containing perfringolysin as an antitumor toxin (Tsuji et al., 2016). The internalization of these minicells was achieved with invasin on the bacterial surface for the treatment of xenograft bladder tumors in mice. In vitro infection with VAX-IP results in the internalization on minicells mediated by invasin.

In this PhD thesis we reduced the process for generation of minicells to just one step by creating minicells already loaded with a protein of interest and expressing a SAs against EGFR on their surface. However, minicells expressing the SA against EGFR presented a weak adhesion to tumor cells expressing EGFR in vitro despite the fast and robust adhesion to live bacteria with the same SA to these cells. When minicells have been reported to be used in vivo, a high dose of minicells ($5x10^8$/animal)

needs to be administered in multiple inoculations in order to see a significant anti-tumor effect, both in subcutaneous and orthotopic tumor models (MacDiarmid and Brahmbhatt, 2011; Tsuji et al., 2016) These data also suggest a poor targeting of minicells to tumor cells in these studies. The number of live bacteria needed for colonization of solid tumors (ca. 10^6-10^7 in the absence of SA) is much lower than the number of minicells administered in vivo with a targeting mAb or invasin. Hence, live bacteria appear to be more appropiate vehicles for tumor targeting with SAs. In addition of their simple and inexpensive production, live bacteria are able to survive and replicate inside the tumor, keeping continuous expression of antitumor molecules. In our study we have determined that a major factor explaining the poor adhesion of minicells to tumor cells in comparison with bacteria (expressing the same SA) is the lack of motility in non-live minicells.

20.4. Importance of flagella for bacterial adhesion

Bacteria need to get in close proximity to initiate attachment to any surface (Berne et al., 2018). Our results have determined that, in addition to this initial contact, bacteria need to establish multiple adhesion sites to become permanently attach to the surface of a mammalian cell. Flagella power the swimming motility that enables the bacteria to approach a solid surface. In addition, flagella are also involved in signal transduction in response to chemical and mechanical inputs, can trigger bacteria cell differentiation and participate in important steps during host infection (Berne et al., 2018).

In this PhD thesis, it has been demonstrated that an active flagellar motility, and not the presence of an inactive flagellar filament, is required for the efficient and permanent adhesion of bacteria to tumor cells. E. coli motA mutants assemble flagella but are unable to attach to cells. Flagellar motility facilitates the initial contact of the bacterium with the cell, which mostly occurs with the bacterial pole, due to bacterial swiming. This is the reason why bacterial "tumble" mutants (ΔcheZ) have a reduced adhesion to cells unless the initial contact is facilitated by a different mechanism (e.g. centrifugation). Importantly, flagellar motility is also needed for the transition to a permanent attachment. We have shown that bacterium needs to adopt a parallel position to the cell surface after the initial contact with the bacterial pole, establishing different binding spots in the main rod body of the bacterium, allowing to remain permanently attached to the mammalian cell even under strong shear forces in fast flow conditions.

From these results we can conclude that the flagella is helping the bacterium to make initial contact with the cell and, subsequently, is twisting the bacterial body which then "traps" the bacterium establising several adhesion spots. The mechanism may find a parallelism in the way insects become trapped in spider webs, since movement of the bug make it become attach in further spots by the web. In addition, we found that this is happening not just in the case of the adhesion through synthetic adhesins, but also with natural adhesins from pathogenic bacteria like invasin for *Yersinia pseudotuberculosis* and type I fimbiae from uropathogenic E. coli. We also found that a Δ*flhDC* mutant of *Salmonella* is not able to invade NRK-fibroblast cells. Hence, our results have implications to understand adhesion of host cells by pathogenic strains and not only for the use of bacteria against cancer. It has been described that the flagellum is an important pathogenicity determinant in numerous pathogens such E.coli, Listeria, Salmonella and Caulobacter, in which their ultimate goal is to invade or attach to the host cell (Chaban et al., 2015) (Berne et al., 2018).

In addition, overexpression of the master flagellar regulator flhDC, which iincrease the number of flagella in the bacterial body, has been found to increase invasion of Salmonella to tumor masses in vitro and colonization of tumors in vivo (Raman et al., 2019) (Thornlow et al., 2015; Toley and Forbes, 2012).

Flagella are described to be key elements from a transient to a permanent bacterial attachment in biofilms (Van Dellen et al., 2008). The proton motive force ($\Delta\Psi$) has been suggested to play a role in this process. When bacteria are swimming in liquid media the $\Delta\Psi$ is maintained at a constant level. When the cell attached to a surface, the flagellar motors cannot rotate and it stops the flow of ions through the motor, resulting in a transient increase of $\Delta\Psi$ (Van Dellen et al., 2008). This may indicate to the bacterium that is close to a surface and initiates the transition to a permanent attachment. For instance, *Vibrio cholera* monolayer formation in abiotic surfaces is inhibited by addition of valinomycin, an antibiotic which suppress the $\Delta\Psi$ (Van Dellen et al., 2008). However, we found that addition of valinomycin does not inhibit the adhesion of *E. coli* to tumor cells.

It is interesting understanding the molecular pathways implicated in flagellar signal transduction. Currents studies indicate that the second messenger cyclic di-GMP (ci-di-GMP) inhibits motility in bacteria (Paul et al., 2010). This dinucleotide is produced by a family of enzymes called diguanylate cyclases (DGCs) and is able to inhibit bacterial motility directly by interaction with the YcgR brake protein in E. coli (Boehm et al., 2010). The transcription of the major DGC in E. coli, DgcZ, is activated by the lipoprotein NlpE in response to surface sensing (Lacanna et al., 2016). YcgR bound to

c-di-GMP is able to stop flagella by interaction with FliM and FliG component of flagella rotor (Paul et al., 2010). In our work we found that overexpression of c-di-GMP in the E. coli WT strain, but not and in the Δ*ycgR* mutant, stopped flagellar motility. However the absence of this brake protein did not have any apparent effect in the adhesion process to tumor cells. *E. coli* Δ*ycgR* strain expressing the SA against EGFR was able to reach and bind tumor cells expressing EGFR on their surface at normal levels and remained permanently attached to the cell.

In conclusions, our observations clearly support the essential role of active flagellar motility in bacterial adhesion to mammalian cells, and highlight the importance of this process for adhesion to tumor cells by synthetic bacteria and also during host cell adhesion and invasion by bacterial pathogens. Our findings also indicate that is not necessary a fast stop of the flagella mediated elevated c-di-GMP levels induced by known surface sensors (i.e.NlpE, DgcZ, YcgR), or and increase in $\Delta\Psi$, for the bacteria to become attached to cells.

Conclusions.

1. The affinity and binding competition of five different nanobodies binding human EGFR has been chracterized. Two nanobody clones showing high affinity and binding to different epitopes on the EGFR molecule have been selected for construction of *E. coli* synthetic adhesins.

2. We have demonstrated the constitutive and stable expression of synthetic adhesins against EGFR on *E. coli* surface after insertion of genetic constucts in the bacterial chromosome. This expression drives the specific adhesion the modified bacterium to human epithelial tumor cell lines expressing EGFR, including bladder and colon carcinomas.

3. We have engineered the inducible expression of invasin from *Yersinia pseudotuberculosis* in the chromosome of *E. coli* bacteria expressing synthetic adhesins against EGFR. Induction of invasin upon bacterial adhesion leads to the specific invasion of tumor cells. Bacterial invasion increase apoptosis of tumor cells but it is not sufficient to cause a massive death of the infected tumor cells.

4. We have found that a bacterial protein cargo can be detected in the cytoplasm of the invaded tumor cells after 24 h of infection, especially when the engineered bacterium carries an auxotrophy for diaminopimelic acid (inducing its lysis in the endosome) and co-expresses LLO from *Listeria monocytogenes*.

5. We have produced non-live *E. coli* minicells expressing synthetic adhesins against human EGFR on their surface and a cargo protein in their cytosol. The adhesion to tumor cells achieved in vitro by these engineered minicells was poor in comparison with live bacteria, despite higher titers and longer incubation times used with minicells.

6. We have found that bacterial motility mediated by flagella is required for the efficient adhesion of *E. coli* bacteria expressing synthetic adhesins to target tumor cells. *E. coli* mutants lacking flagella or having a non-motile flagella are

not able to bind to target tumor cells despite the expression of the synthetic adhesins. This adhesion defect of non-motile bacteria is not compensated by bacterial movement in an unidirectional flow or by centrifugation to force cell contact.

7. At least one of the flagellar rotation movements, CCW or CW, leading to linear swimming or tumbling motility of the bacterium, is required to trigger the permanent adhesion of bacteria with synthetic adhesins upon an initial cell contact. Tumbling mutants need to force this initial cell contact (e.g. by centrifugation) due to their lack of a linear swimming.

8. Real-time live microscopy indicate that an individual *E. coli* bacterium must establish simultaneously several adhesion points to the plasma membrane of the tumor cell in order to produce a permanent adhesion event. After an initial cell-contact, the flagellar activity is needed for the generation of local bacterial movements that allow the establishment of additional adhesion points between the bacterium and the tumor cell. We do not observe movement of bacteria attached to cells, suggesting the stop of the flagellar motor after a permanent attachment is established.

9. Neither the absence of the flagellar "brake" protein YcgR, nor of NlpE and DcgZ components of the c-di-GMP-signalling pathway activating YcgR, have any significant effect on the adhesion of bacteria to tumor cells. In addition, the adhesion process in not affected by the dissipation of the electrochemical potential of the bacterial plasma membrane mediated by Valinomycin.

10. We found evidence that the dependence of the flagella for bacterial adhesion also occurs in the case of natural adhesins expressed in *E. coli* (i.e. invasin and type I fimbriae) and for the adhesion and invasion of mammalian cells by *Salmonella enterica* sv. *typhimurium*.

Conclusiones

1. Hemos caracterizado la afinidad y competición de cinco anticuerpos monodominio que unen el EGFR humano. Seleccionamos dos "nanobodies" con alta afinidad y reconocimiento de epítopos distintos dentro de EGFR para constuir adhesinas sintéticas de E. coli.
2. Hemos demostrado la expresión constitutiva y estable de las adhesinas sintéticas frente EGFR en la superficie de E.coli tras la inserción de los constructos genéticos en el cromosoma bacteriano. La expresión de las adhesinas permitió dirigir la adhesión específica de las bacterias modificadas a células tumorales que expresan EGFR, incluyendo carcinomas de vejiga y colon.
3. Hemos generado la expresión inducible de invasina de Yersinia pseudotuberculosis en el cromosoma de bacterias E. coli que expresaban las adhesina sintéticas frente a EGFR. La inducción de la invasina tras la adhesión bacteriana permite una invasión específica de las células tumorales. La invasión bacteriana incrementa la apoptosis de las celulas tumorales pero es insuficiente para causar una muerte masiva de las células tumorales infectadas.
4. Hemos comprobado que una proteína "cargo" expresada en la bacteria puede ser detectada en el citoplasma de la célula tumoral invadida 24 horas tras la infección, especialmente cuando la bacteria es auxótrofa para diaminopimélico (lo que induce su lisis en el endosoma) y coexpresa LLO de Listeria monocytogenes.
5. Hemos producido minicélulas no vivas de E. coli que expresan SAs frente a EGFR en su superficie y una proteína "cargo" en el citosol. La adhesión a células tumorales lograda in vitro por estas minicélulas modificadas fue muy escasa en comparación con las bacterias vivas, incluso utilizando altas dosis de minicélulas y largos tiempos de incubación.
6. La motilidad bacteriana mediada por el flagelo es requerida para una adhesión efectiva de la bacterias E. coli que expresan SA contra EGFR. Los mutantes de E. coli que no tienen flagelo, o poseen un flagelo no motil, no son capaces de unirse a las células tumorales diana. Este defecto en la adhesión no puede ser compensado con el movimiento de la bacteria en un flujo unidireccional o mediante centrifugación para forzar el contacto entre la bacteria y la célula.

7. Al menos uno de los movimientos de rotación flagelar, CCW o CW, que dirigen una natación lineal o rotatoria, es sufiente para que la bacteria pase de una adhesión transitoria a una permanente una vez ha contactado con la célula diana. Los mutantes que nadan en movimiento rotatorio necesitan una fuerza inicial que dirija a la bacteria contra la célula (ej. centrifugación) debido a la falta de dirección en el movimiento.

8. La microscopía a tiempo real con células vivas indica que una bacteria E. coli debe establecer simultáneamente varios puntos de adhesion a la membrana de la célula tumoral para que se produzca una adhesión permanente. Tras el primer contacto bacteria-célula tumoral, la actividad del flagelo es necesaria para la generación de movimientos locales de la bacteria que permiten el establecimiento de puntos de adhesión adicionales. Tras esta adhesión permanente no se observa movimiento de la bacteria, lo que sugiere una parada de la rotación del flagelo.

9. La falta de proteína "freno" del flagelo YcgR, o de las proteínas de la ruta de señalización de c-di-GMP que activa YcgR, no son necesarias para la adhesión de la bacteria a la célula. El proceso de adhesión tampoco se ve afectado por la disipación de potencial de membrana debido a la adición del antibiótico Valinomicina.

10. Hemos comprobado que la dependencia del flagelo para la adhesion bacteriana también ocurre en el caso adhesinas naturales expresadas en E. coli como la invasina de Yersinia pseudotuberculosis o las fimbrias tipo I de E. coli uropatógenas, así como para la adhesión e invasión de células de mamífero por Salmonella enterica sv. typhimurium.

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